SELECTIVE EFFECT OF SODIUM IONS ON A COMPONENT OF THE STIMULATION-INDUCED INCREASE IN TRANSMITTER RELEASE AT THE FROG NEUROMUSCULAR JUNCTION

By

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

SELECTIVE EFFECT OF SODIUM IONS ON A COMPONENT OF THE STIMULATION-INDUCED INCREASE IN TRANSMITTER RELEASE AT THE FROG NEUROMUSCULAR JUNCTION

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Repetitive stimulation of presynaptic nerve terminals often leads to an increase in the amount of transmitter released by a nerve impulse. Accumulation of Ca^{2+} and/or Na^{+} ions within the nerve terminal has been proposed to explain this increase in release. This dissertation describes the results of experiments performed to characterize the roles of these ions in stimulation-induced increases in neurotransmitter release at the frog (Rana pipiens) neuromuscular junction.

Standard extra- and intracellular recording techniques were used to record end-plate potentials (EPPs) from the frog sartorius nerve-muscle preparation. The nerve was conditioned using trains of 10 impulses at 20 impulses/s in order to examine the effect of repetitive stimulation on V(t), the stimulation-induced change in EPP amplitude.

Reduction of the external Na^{+} concentration produced a concentration-dependent and reversible increase in V(t). The Na^{+} effect on V(t) appeared to occur early during the

conditioning train and to reach a plateau before the end of the train, consistent with an effect of Na^+ on an early component of increased release. Monensin, a Na^+-selective ionophore which acts to increase intracellular Na^+ concentrations, had a qualitatively similar effect on V(t), suggesting that the Na^+ effect is mediated through a mechanism which is dependent on the driving force for Na^+ entry into the nerve terminal. The magnitude of the Na^+ effect was dependent upon the external concentration of Ca^{2+}. These observations are consistent with the hypothesis that a Na^+/Ca^{2+} exchange mechanism may be involved in stimulation-induced increases in release at the neuromuscular junction.

Initiation of evoked transmitter release has traditionally been attributed to Ca^{2+} entering the nerve terminal from the extracellular fluid during the action potential. However, intracellular Ca^{2+} has also been proposed to play a role in evoked release. To examine this hypothesis, nerve-muscle preparations were exposed to ouabain, a Na^+/K^+ pump inhibitor which secondarily elevates intracellular Ca^{2+}. Following treatment with ouabain, evoked release could be maintained in the virtual absence of external Ca^{2+}. This finding supports the hypothesis that intracellular Ca^{2+} may play a role in the process of evoked transmitter release.

CHAPTER 1

INTRODUCTION

Rapid communication of electrical signals between most neurons and other excitable cells is dependent upon the process of chemical synaptic transmission. In this process, an electrical impulse is conducted into the presynaptic nerve terminal, initiating events which lead to the release of a specific chemical substance, or neurotransmitter, from the nerve terminal. The neurotransmitter then diffuses across the cleft which separates the presynaptic and postsynaptic cells and binds to specific receptors on the postsynaptic cell surface, producing a post-synaptic potential, an electrical response in the membrane of the postsynaptic cell (see Kuffler et al., 1984). Because chemical synapses play an essential role in the nervous system, understanding the mechanisms underlying synaptic function is of great importance in the understanding of nervous system organization and function.

Although much is known about the mechanisms of impulse propagation into the nerve terminal, and about the responses induced in the postsynaptic membrane by a neurotransmitter, relatively little is understood about the process of release of neurotransmitter substances from the nerve terminal. It is known that transmitter substances

are released from the presynaptic nerve terminal in packets, or quanta, containing similar amounts of neurotransmitter (del Castillo and Katz, 1954a; Boyd and Martin, 1956). There is a great amount of evidence that the release of a quantum of transmitter results from exocytosis of the contents of a transmitter-containing synaptic vesicle into the synaptic cleft (e.g., Heuser et al., 1979), although alternative mechanisms such as carrier proteins and transmitter-specific pores or gates in the cell membrane have been proposed (Tauc, 1982). There is also good evidence that evoked quantal transmitter release is generally initiated by the entry of calcium ions into the nerve terminal (Katz and Miledi, 1967a,b; Llinas and Nicholson, 1975). Transmitter release appears to be proportional to the third or fourth power of the calcium concentration at or near the release site, an observation which has been interpreted as evidence for a co-operative interaction of three or four calcium ions on the release mechanism (e.g., Dodge and Rahamimoff, 1967). Despite these advances in our understanding of the release process, however, it is not known what events occur to initiate quantal release after calcium enters the nerve terminal.

In addition to evoked quantal release, quanta of neurotransmitter are released spontaneously from the presynaptic nerve terminal at a variety of central and peripheral synapses including frog and crayfish neuromuscular junctions and frog spinal motoneurons (Fatt and Katz, 1952; Dudel and

Kuffler, 1961; Katz and Miledi, 1963). At low frequencies of spontaneous quantal release, the timing of release events appears to be random (reviewed in Martin, 1966). At the neuromuscular junction, the amplitude of the miniature end-plate potential, or postsynaptic response to a single quantum of transmitter, has been shown to be virtually identical to the amplitude of the smallest evoked end-plate potential, or unit potential (Fatt and Katz, 1952; Martin, 1966). The marked similarity between these two end-plate responses led del Castillo and Katz (1954a) to propose that the end-plate potential resulted from the synchronized release of one or more quanta during depolarization of the presynaptic nerve terminal (the 'quantal hypothesis' of transmitter release).

A continuous, nonquantal release of transmitter which results in a small (about 40 uV), tonic depolarization of the end-plate has been described at frog and rat neuromuscular junctions (Katz and Miledi, 1977a). Such nonquantal acetylcholine release may represent as much as 98% of the total amount of acetylcholine released by the resting nerve terminal (Katz and Miledi, 1977a; Vizi and Vyskocil, 1979). The function of this spontaneous, nonquantal release of transmitter is unknown, although it has been suggested to have trophic effects on the neuromuscular synapse (e.g., Vizi and Vyskocil, 1979). Nonquantal release of transmitter is unlikely to contribute to the generation of the end-plate potential, however, as its effect on the membrane

potential of the muscle fiber is almost two orders of magnitude smaller than the response to a single quantum of transmitter (Katz and Miledi, 1977a). Furthermore, no increase in the magnitude of nonquantal release could be detected during nerve stimulation under conditions of reduced extracellular Ca^{2+} concentration which were sufficient to abolish evoked transmitter release (Katz and Miledi, 1981). The discussions of transmitter release in this dissertation will be restricted to properties of quantal transmitter release.

Another intriguing and widespread property of synapses is the modulation of the amount of transmitter released by a nerve impulse as a result of previous synaptic activity. Stimulation-induced increases in synaptic responses at peripheral synapses have been shown to result from increases in transmitter release (Liley and North, 1953; del Castillo and Katz, 1954b; Martin and Pilar, 1964; Mallart and Martin, 1967). Increases in synaptic responses during repetitive stimulation have also been described at a number of central synapses (Curtis and Eccles, 1960; Porter, 1970; McNaughton, 1980; Hess et al., 1987). As the nervous system uses trains of impulses to transmit signals, understanding the effect of repetitive stimulation on transmitter release is particularly relevant to the understanding of nervous system function. For instance, changes in the magnitude of the synaptic response as a result of repeated activation of a synapse have been suggested to

underlie many aspects of learning and memory (e.g., Hebb, 1949). Activity-induced modification of synaptic transmission may also play a major role in nervous system disease processes such as the epileptic seizure focus, in which abnormalities of synaptic function such as triggering of prolonged bursts of action potentials and paroxysmal depolarizing shifts in postsynaptic membrane potentials have been described (reviewed in Taylor, 1988).

As with the mechanism of transmitter release itself, the mechanisms responsible for changes in transmitter release due to repetitive stimulation are still poorly understood. Katz and Miledi (1965, 1968) have suggested that the phenomenon of increased release may occur as a result of the accumulation of calcium within the nerve terminal during repetitive stimulation. However, this hypothesis cannot easily account for all of the processes of increased release which have been described at the neuromuscular junction (Zengel and Magleby, 1982).

Understanding the mechanisms responsible for changes in transmitter release due to repetitive stimulation may give some insight into the mechanism of the release process itself. Any model proposed to describe the release process will have to account for the numerous properties of release which have been reported. Among these are the calcium dependence of release (Dodge and Rahamimoff, 1967), the divalent cation selectivity of release ($Ca^{2+} > Sr^{2+} > Ba^{2+}$; Miledi, 1966; Blioch et al., 1968; Augustine and Eckert,

1984), the very short delay for initiation of release (less than 200 us; Llinas et al., 1981), and the occurrence of stimulation-induced changes in release. The following chapters will describe the results of investigations of two such properties, the increase in transmitter release due to repetitive stimulation and the calcium dependence of release, as a means of better understanding the release process.

CHAPTER 2

SELECTIVE EFFECT OF SODIUM IONS ON STIMULATION-INDUCED CHANGES IN TRANSMITTER RELEASE

Introduction

At the frog neuromuscular junction, repetitive stimulation, under conditions of low quantal content, results in increases in evoked quantal release during the stimulus train. This increase in evoked release has been attributed to accumulation of "residual" calcium or a calcium-activated factor within the nerve terminal, which is only slowly removed from the cytoplasm after passage of a nerve action potential (Katz and Miledi, 1968; Rahamimoff, 1968; Zucker and Lara-Estrella, 1983). The influx of calcium accompanying the subsequent nerve action potential would add to the small increase in calcium persisting from the previous action potential. As transmitter release appears to be related to the third or fourth power of the calcium concentration at or near the release site, even a small increase in residual cytosolic calcium could lead to a significant increase in release (Dodge and Rahamimoff, 1967; Charlton et al., 1982).

The stimulation-induced increase in release at the frog neuromuscular junction appears to have at least four components: two short-acting components of facilitation (Mallart

and Martin, 1967; Magleby, 1973; Younkin, 1974), a long-term process called potentiation (Rosenthal, 1969; Magleby and Zengel, 1975), and an intermediate component termed augmentation (Magleby and Zengel, 1976; Erulkar and Rahamimoff, 1978; Zengel and Magleby, 1982). Although residual intracellular calcium has been suggested to play a role in each of these processes, their ionic dependence is not clearly understood. Many authors have suggested that increases in intracellular sodium concentrations may contribute to one or more of the processes of increased release, either by a direct effect of intraterminal sodium or by an indirect effect of sodium on intracellular calcium concentrations (e.g., Rahamimoff et al., 1980).

Although the experiments of Katz and Miledi (1967b) have shown that Na^+ entry into the nerve terminal is not necessary for release to occur, there is a great deal of evidence that manipulations known to affect intracellular Na^+ can modulate release and stimulation-evoked changes in release. Blockade of the Na^+/K^+ pump by ouabain, which elevates $[Na^+]_i$, can increase evoked release (Birks and Cohen, 1968b, Baker and Crawford, 1975). Introduction of Na^+ into motor nerve terminals using Na^+-filled liposomes increases both evoked and spontaneous transmitter release at the frog neuromuscular junction (Rahamimoff et al., 1978). Accumulation of $[Na^+]_i$ has been implicated in the process of potentiation at frog neuromuscular junctions (Lev-Tov and Rahamimoff, 1980; Meiri et al., 1981), while

introduction of Na^+ into the nerve terminal with the Na^+ ionophore monensin increases a slow time course process called "long-term facilitation" at the crayfish neuromuscular junction (Charlton et al., 1980; Atwood et al., 1983). However, there has been no systematic investigation of the effects of manipulations of $[Na^+]_i$ on shorter time course components of increased release.

Whether Na^+ ions affect the release mechanism directly, or act indirectly by increasing intracellular $[Ca^{2+}]$, is unknown. Originally it was proposed that Na^+ may compete with Ca^{2+} for entry into the nerve terminal (Colomo and Rahamimoff, 1968), although this effect may be insignificant at low Ca^{2+} concentrations in the extracellular medium. Decreases in external $[Na^+]$ or increases in internal $[Na^+]$ may also alter intracellular $[Ca^{2+}]$ by inhibiting Ca^{2+} extrusion via the sodium-calcium exchanger, which is driven by the electrochemical gradient for Na^+ across the plasma membrane (e.g., Blaustein and Oborn, 1975; Dipolo and Beauge, 1983). Elevated internal $[Na^+]$ has been suggested to trigger release of Ca^{2+} from intracellular stores (Lowe et al., 1976; Rahamimoff et al., 1980).

In this study, reductions in extracellular $[Na^+]$ are shown to selectively increase an early component of the stimulation-induced increase in transmitter release at the frog neuromuscular junction under conditions of reduced quantal content. Addition of monensin, an ionophore known to increase intracellular $[Na^+]$ (Pressman and Fahim, 1982),

has a qualitatively similar effect on increased release. The magnitude of the Na^+ effect on this early component of increased release appears to be dependent on extracellular $[Ca^{2+}]$. These observations suggest that a sodium-calcium exchange mechanism may be involved in stimulation-induced increases in transmitter release at the frog neuromuscular junction. Portions of this work have been published in abstract form (Mosier et al., 1986; Mosier and Zengel, 1987).

Materials and Methods

Experimental Procedures

The results reported in this study are based upon observations made on more than 120 sartorius nerve-muscle preparations from the frog Rana pipiens. Healthy frogs of 2 to 2 1/2 inch length were obtained from Nasco and Charles Sullivan and housed in a basin at 20-22 ^{o}C prior to usage. Frogs were decapitated and the sartorius muscle with the attached nerve was dissected in normal Ringer's solution with special care taken to minimize trauma to the preparation. The nerve to the sartorius muscle was cut at the point of its separation from the sciatic nerve, approximately 4-5 mm from the entry site of the sartorius nerve into the muscle. The sartorius muscle was stretched slightly by pinning the connective tissue at the borders of the muscle to wax at the bottom of the recording chamber.

The muscle nerve was stimulated with a fluid suction electrode (Dudel and Kuffler, 1961). One end of a PE-60 polyethylene tube of 0.76 mm inside diameter (Clay Adams, no. 7416) was drawn after gentle heating to an inside diameter of approximately 0.3 mm. The other end of the tube was connected to a 10 cc syringe, which was used to draw the nerve and bathing solution into the tapered end. The nerve was stimulated by the passage of a supramaximal stimulus of 0.01-0.1 ms duration between a silver-silver chloride (Ag-AgCl) wire inserted into the suction electrode and a similar Ag-AgCl wire in the bath.

After observing a muscle twitch upon stimulation of the sartorius nerve, which verified that action potential conduction was intact in the nerve, the bathing medium was changed to a solution containing 5 mM Mg^{2+} and reduced concentrations of Ca^{2+} (0.4-0.6 mM) in order to reduce the quantal content, or number of quanta of transmitter released per nerve impulse (del Castillo and Stark, 1952; del Castillo and Katz, 1954a). Under these conditions, the mean quantal content of release was typically 0.2 to 2 for the first impulse in a conditioning train, and rarely exceeded 3.5 during a 10 impulse train. Nerve stimulation under conditions of reduced quantal content does not evoke a muscle contraction, thereby allowing stable recordings of end-plate responses to be made. Reduction of the quantal content also minimizes the effect of stimulation-induced depression of transmitter release, which has been attrib-

uted to depletion of the amount of transmitter available for release (e.g., Liley and North, 1953; Thies, 1965; Elmqvist and Quastel, 1965, Mallart and Martin, 1968). Furthermore, indirect methods of estimating quantal content (discussed later in this section) are based on the assumption of a Poisson distribution of quantal content, which is valid only at low probabilities of release (corresponding to average quantal contents of less than 4; del Castillo and Katz, 1954a; Martin, 1966).

Extracellular recordings of end-plate potentials (EPPs) were obtained with a surface electrode from end-plate regions of the frog sartorius muscle. Under conditions of reduced quantal content, changes in the amplitudes of extracellularly recorded EPPs have been shown to be a good measure of changes in transmitter release (Mallart and Martin, 1967; Magleby, 1973). The surface electrode was constructed from PE-60 tubing similar to that used for the stimulating electrode. A 3.5 cm length of tubing was heated gently at one end to form a smooth flange around the tube opening, and a Ag-AgCl wire was inserted into the tube from the other end. The surface electrode was filled with the bathing solution used in the experiment, and the flanged end was visually positioned close to an end-plate region of the muscle. End-plate potentials were recorded differentially between the Ag-AgCl wire in the surface electrode and a similar Ag-AgCl wire placed in the bath. The electrode was moved over the surface of the muscle to

locate the position of the maximum end-plate response to nerve stimulation. After placing the electrode tip very close to the muscle at a site of maximal end-plate response, the electrode was not shifted during the course of the experiment.

The nerve to the sartorius muscle was stimulated using a Grass S48 or S88 stimulator and a Grass SIU5 stimulus isolation unit. To rule out increases in transmitter release due to recruitment of axons, preparations were stimulated at five times the voltage required to evoke a maximally sized single EPP. For most experiments, trains of 10 impulses applied at a stimulus frequency of 20 impulses/s were used to condition the nerve. The conditioning trains were delivered to the nerve once every 55 s. In a few experiments, paired pulses were delivered to the muscle nerve every 40 s. These intervals should be of sufficient duration to allow the nerve terminal to recover to a control state between trains or pairs of conditioning stimuli (Magleby and Zengel, 1975).

Extracellular responses obtained with the surface electrode were amplified with a Grass P511 A.C. preamplifier and displayed on a Tektronix model 5113 dual beam storage oscilloscope. Amplified responses were filtered at 10 kHz. In most experiments, the responses to 4 to 128 trains of impulses delivered under each experimental condition were averaged for analysis using a Nicolet 1170 signal averaging computer. End-plate potential amplitudes were measured

directly from the digitized data displayed on the screen of the signal averaging computer during the course of the experiment. In some experiments, amplified responses from the surface electrode were digitized using the 12-bit analog-to-digital converter of a MINC-11 computer which sampled and recorded EPP amplitudes for subsequent averaging and analysis (Magleby and Zengel, 1976). As the absolute magnitude of the end-plate potential measured with a surface electrode may vary due to electrode placement and the location within the muscle of the end-plates sampled, all EPP amplitudes within an experiment were normalized to the average amplitude of the first EPP of trains recorded in the control bathing medium. Comparisons of EPP amplitudes between preparations were made on the basis of these normalized data.

Intracellular recordings were performed using glass microelectrodes filled with 3M KCl, with tip resistances of 5-15 megohms. Microelectrodes were pulled on a David Kopf model 720 vertical pipette puller using glass capillaries obtained from World Precision Instruments (WPI #TW150F). End-plates were located by inserting a microelectrode into muscle fibers in a visually identified end-plate region. The microelectrode was inserted along the length of a muscle fiber to the position which gave the maximum EPP amplitude and minimum EPP rise time (typically less than 1.0 ms), and at which the mean miniature end-plate potential (MEPP) amplitude was at least 0.4 mV. Although the

majority of end-plates of twitch muscle fibers in the adult frog are innervated by only a single axon, polyneuronal innervation of end-plates has been demonstrated (e.g., Trussell and Grinnell, 1985). To prevent effects due to recruitment of nerve fibers, the nerve to the sartorius was stimulated at 5 times the threshold voltage required to evoke an end-plate response. No differences in average EPP amplitude were noted at different stimulation intensities above threshold, suggesting that a significant contribution of polyneuronal innervation was unlikely among the end-plates sampled in this study.

End-plate potentials recorded using intracellular techniques were typically less than 1.5 mV in amplitude in the absence of repetitive stimulation and seldom exceeded 3 mV during the conditioning trains. No corrections were made for nonlinear summation of unitary EPPs, since experimental evidence suggests that such corrections are unnecessary if EPP amplitudes are less than 10 mV (McLachlan and Martin, 1981). Because of the conditions of reduced quantal content in these experiments, quantal fluctuation was usually too great to estimate responses from single preparations in which intracellular recording techniques were employed. Consequently, data from a number of preparations were averaged for analysis.

The standard bathing solution had the following composition (in mM): NaCl 116, KCl 2, $CaCl_2$ 1.8, HEPES 2, glucose 5. This solution was modified by reducing $[Ca^{2+}]$ to

0.4-0.7 mM and adding 5 mM Mg^{2+} to decrease transmitter release. Osmolality was maintained by reducing NaCl. The pH was adjusted to 7.2-7.4 using NaOH. Alterations of pH of the bathing medium within this range did not appear to have any obvious effect on control EPP amplitude or on stimulation-induced increases in EPP amplitude. Experiments were performed at 20-22 ^{o}C. Solution changes were performed with Pasteur pipettes and typically required 1-2 min to complete. Amiloride, N-methylglucamine, ouabain, and monensin were obtained from the Sigma Chemical Company.

Changes in the osmolality of the bathing medium have been shown to have an effect on transmitter release (Fatt and Katz, 1952; Furshpan, 1956). In solutions in which extracellular Na^{+} was reduced by the removal of NaCl, osmolality was maintained by the addition of sucrose in the proportion: 183 mM sucrose = 100 mM NaCl (Birks and Cohen, 1968b). Solutions prepared to control for the effect of Cl^{-} removal employed equimolal substitution with N-methylglucamine chloride to maintain osmolality. In experiments performed to ascertain the effect of increased extracellular [Na^{+}] on increases in release, osmolality was increased in the normal [Na^{+}] bathing media by adding sucrose. The order of exposure to different bathing solutions was varied to control for possible long-term drift that can occur in a preparation over time.

Data Analysis

The fractional change in EPP amplitude, V(t), is given by the following formula:

$$V(t) = EPP(t)/EPP(0) - 1 \qquad \text{(eqn. 2-1)}$$

where EPP(t) represents the end-plate potential amplitude at time t, and EPP(0) represents the control (unfacilitated) EPP amplitude in the absence of repetitive stimulation. A similar formula,

$$V_m(t) = m(t)/m(0) - 1 \qquad \text{(eqn. 2-2)}$$

is used to describe changes in quantal content (m) with repetitive stimulation.

Three methods of estimating quantal content were used (del Castillo and Katz, 1954a, Martin, 1966). Quantal content could be calculated directly using the equation

$$m = \overline{EPP}/\overline{MEPP} \qquad \text{(eqn. 2-3)}$$

where $\overline{EPP}$ and $\overline{MEPP}$ are the mean EPP and MEPP (miniature end-plate potential) amplitudes, respectively. Indirect estimates of quantal content were obtained from the method of coefficient of variation and the method of failures as shown in the equations below:

$$m = 1/(CV)^2 \qquad \text{(eqn. 2-4)}$$

$$m = \ln (N/F) \qquad \text{(eqn. 2-5)}$$

where CV is the coefficient of variation, N is the total number of stimulations and F is the number of times a stimulation failed to evoke an EPP (del Castillo and Katz, 1954a). Data were discarded from analysis if the quantal content estimates obtained by the methods described above differed by more than 15%. Obtaining consistent estimates of quantal content was a particular problem under conditions of reduced external $[Na^+]$, which leads to a reduction of MEPP amplitude (Fatt and Katz, 1952) and an increase in quantal content (e.g., Birks and Cohen, 1965, 1968b). Both of these effects of reduced $[Na^+]_o$ reduce the accuracy of the above methods of estimating quantal content (e.g., del Castillo and Katz, 1954a).

In most experiments, differences between means were tested for statistical significance using Student's t tests. In the series of experiments conducted to assess the effect of increased external $[Na^+]$ on V(t), the increased variability of V(t) introduced by increases in osmolality precluded the use of parametric tests of statistical significance (see Results). In this situation, a nonparametric test, the Wilcoxon rank-sum test for paired experiments, was used to test for significant differences between population distributions (Mendenhall and Scheaffer, 1973).

In addition to statistical testing of data collected at discrete times during the conditioning trains, increases in V(t) during 10 impulse conditioning trains were modeled as described in Chapter 3. Differences between the model parameters used to describe V(t) during 10 impulse trains were also assessed for statistical significance in order to provide an additional test of the significance of effects described in this chapter. These results will be discussed in detail in Chapter 3.

Results

Effect of Reducing Extracellular Sodium Concentration on V(t)

Reduction of the Na^+ concentration of the solution bathing the frog sartorius neuromuscular junction increased both the control EPP amplitude (the amplitude of the first EPP in a train) and the magnitude of the increase in EPP amplitude which normally occurs during repetitive stimulation under conditions of reduced quantal content. These effects are illustrated in Figure 2-1, which shows EPPs recorded during 10 impulse conditioning trains applied at a stimulus frequency of 20 impulses/s in the presence of normal levels of extracellular Na^+ (also referred to as '100% Na^+'; Figure 2-1A) and following reduction of $[Na^+]$ in the bathing medium to 33% of normal (also referred to as '33% Na^+'; Figure 2-1B). In this experiment, the control EPP amplitude was increased by 82% in the 33% Na^+ Ringer's solution. An increase in control EPP amplitude in 33% Na^+

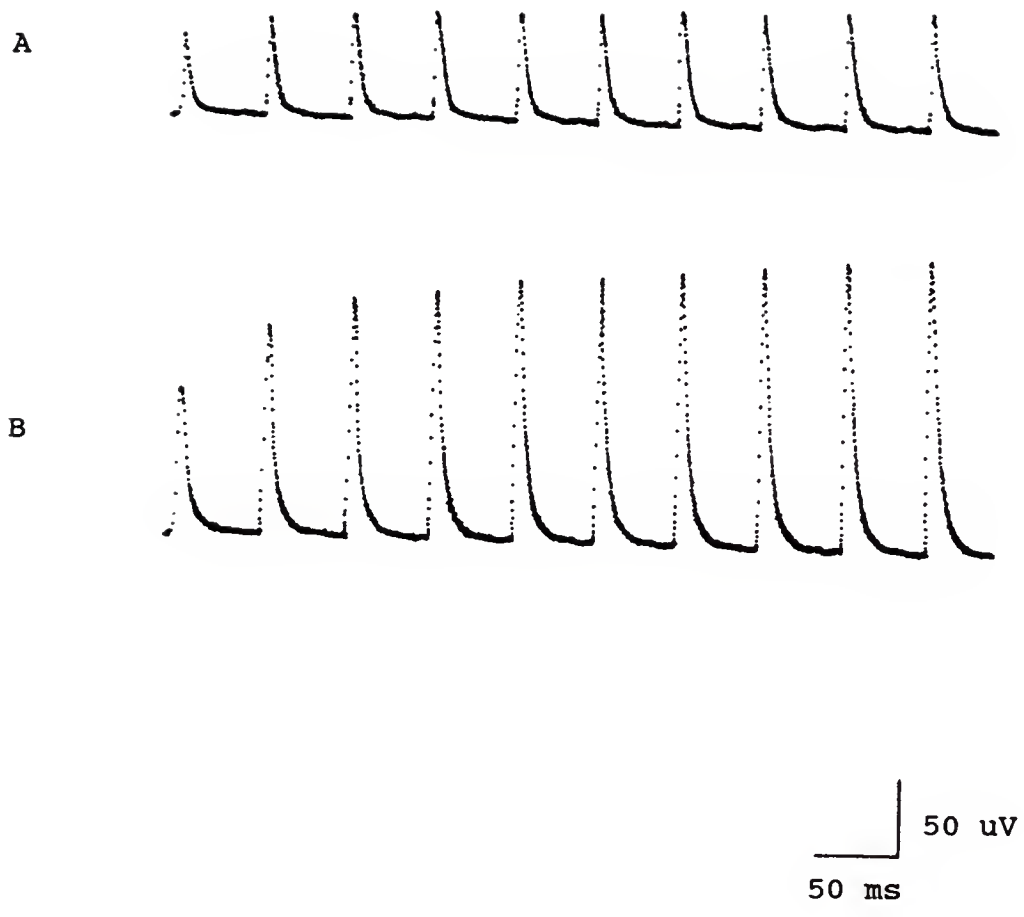


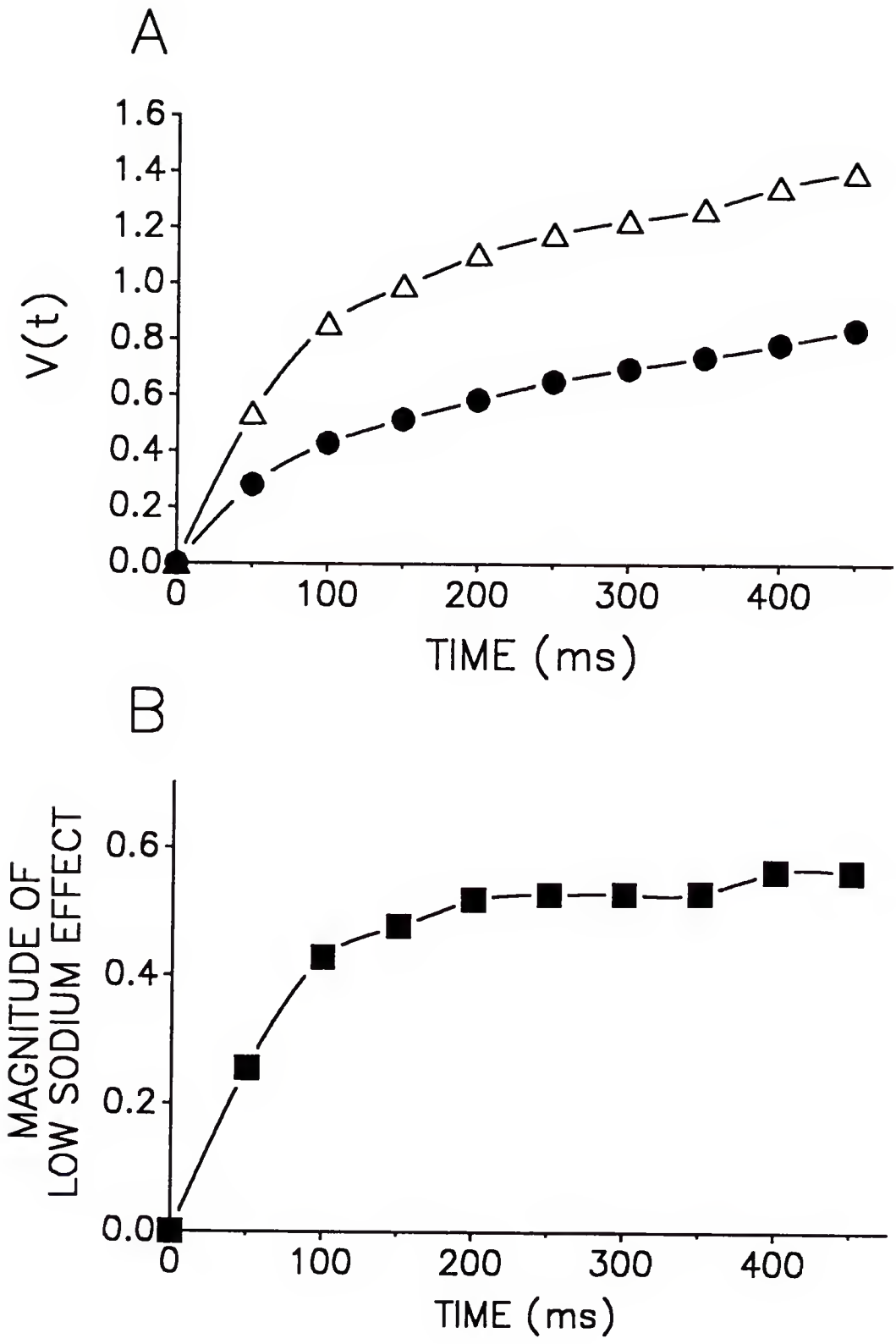
FIGURE 2-1. Effect of reduced external [Na^+] on end-plate potentials recorded extracellularly from the frog sartorius nerve-muscle preparation during 10 impulse conditioning trains. Each record is traced from a photograph of the averaged responses from 32 trains of suprathreshold stimuli delivered to the muscle nerve. Bathing media contained 0.4 mM Ca^{2+}. The preparation was sequentially exposed to bathing media containing 100% of normal [Na^+] (A) and 33% of normal [Na^+] with osmolality maintained by sucrose substitution (B). Both recordings were made at the same voltage gain.

solutions was seen in 46 of 47 experiments. Control EPP amplitudes measured in 33% Na^+ solutions were an average of 2.5 times greater than control EPP amplitudes measured in 100% Na^+ solutions. An increase in EPP amplitude under conditions of reduced extracellular [Na^+] has been described previously at the frog neuromuscular junction, and shown to be due to an increase in quantal content, or the average number of quanta of transmitter released per impulse (Birks and Cohen, 1965; Kelly, 1965; Colomo and Rahamimoff, 1968).

In addition to the effect of reduced external [Na^+] on control EPP amplitude, there was a greater stimulation-induced increase in EPP amplitude during 10 impulse trains recorded in low-[Na^+] bathing solutions. This latter effect is more easily seen in Figure 2-2A, which presents plots of V(t), the fractional increase in EPP amplitude (equation 2-1 in Materials and Methods), as a function of time during 10 impulse conditioning trains.

In the presence of normal levels of external Na^+ (filled circles), V(t) was 0.28 at the time of the second impulse (V(50 ms)), and reached a value of 0.84 by the tenth impulse (V(450 ms)). When the concentration of Na^+ in the bathing solution was reduced to 33% of normal (open triangles), V(50 ms) increased to 0.54, and V(t) reached a value of 1.40 by the tenth impulse. The time course of the Na^+ effect on V(t) during the 10 impulse train is more clearly seen in Figure 2-2B, which shows the difference in

FIGURE 2-2. Effect of reduced extracellular [Na^+] on V(t), the fractional increase in EPP amplitude during 10 impulse trains. (A) Data from a single representative experiment performed at a bath concentration of 0.6 mM Ca^{2+}. Bathing solutions contained 100% of normal [Na^+] (filled circles) or 33% of normal [Na^+] (open triangles), with osmolality maintained by the addition of sucrose. (B) Time course of the increase in V(t) attributable to reduced external [Na^+]. Data are from the same experiment as (A), with the ordinate representing the difference between V(t) measured at 33% and 100% of normal external [Na^+].


A
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
V(t)
0
100
200
300
400
TIME (ms)
B
0.6
0.4
0.2
0.0
MAGNITUDE OF
LOW SODIUM EFFECT
0
100
200
300
400
TIME (ms)

V(t) between the trains of EPPs depicted in Figure 2-2A. The increase in V(t) seen in the low [Na^+] medium appears early during the 10 impulse train and approaches a plateau level by the end of the train. A qualitatively similar increase in V(t) during exposure to 33% Na^+ bathing media was seen in 46 of 47 preparations. The remarkably consistent occurrence and time course of this effect of reduced external Na^+ on V(t), presumably reflecting an effect on an underlying component of stimulation-increased release, will be analyzed in greater detail in the subsequent sections of this chapter.

Specificity of the Effect of Extracellular Sodium Reduction

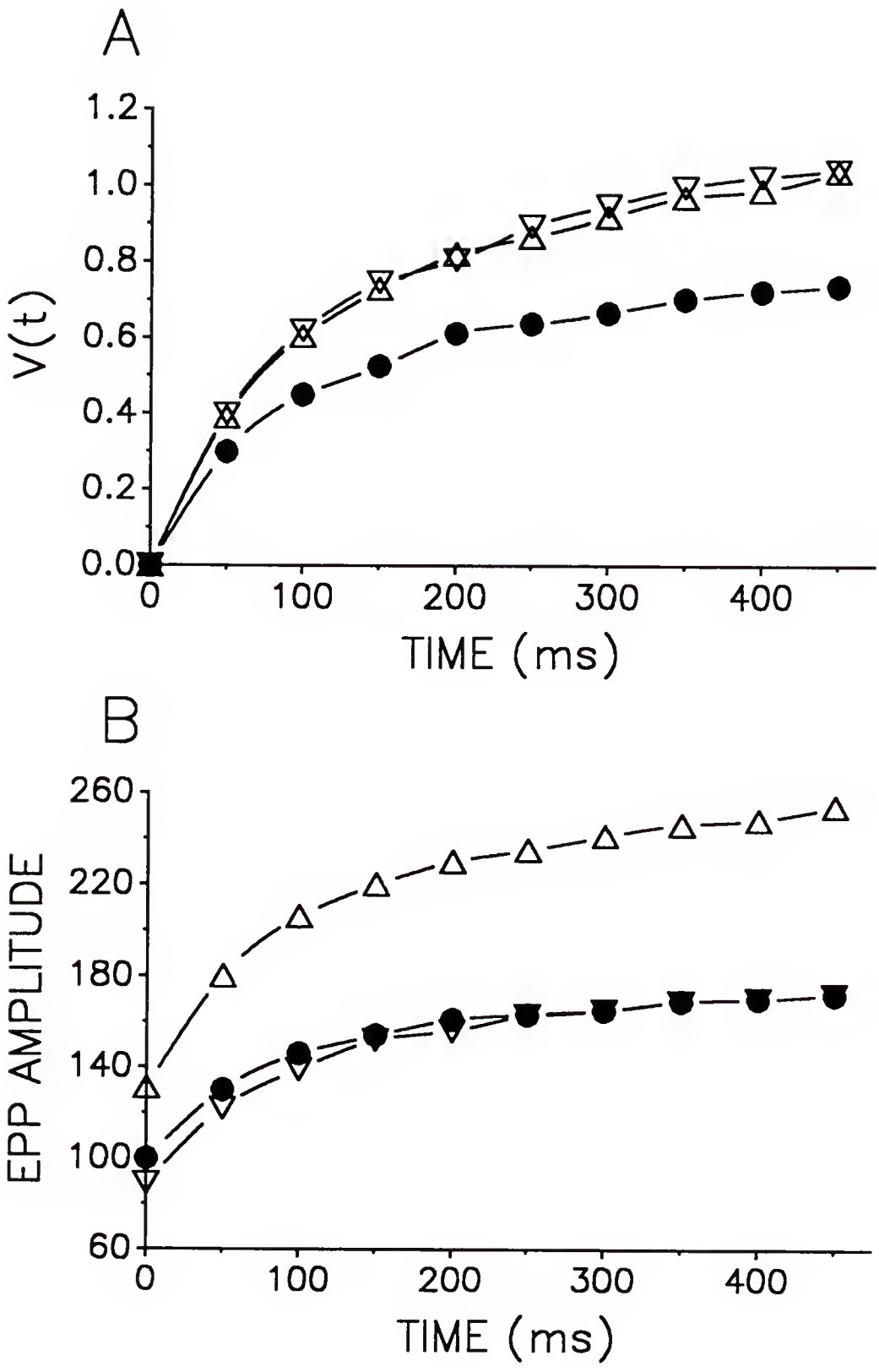
In the experiments described in the preceding section, NaCl was removed from the bathing solutions, resulting in decreases in both [Na^+] and [Cl^-]. To rule out effects on V(t) of changes in extracellular Cl^-, sodium acetate was substituted for NaCl to reduce extracellular [Cl^-] to 33% of the normal concentration. Substitution of acetate for Cl^- had little or no effect on control EPP amplitude. In 4 of 4 experiments, no discernible effect on V(50 ms) of reduced extracellular [Cl^-] was seen (mean $\pm$ S.D., 0.237 ± 0.011, 100% NaCl; 0.231 ± 0.020, 33% Cl^-). In acetate-containing media, there was a small but statistically insignificant increase in V(t) near the end of the 10 impulse train (data not shown). In these same experiments, V(50 ms) was substantially increased by reduction of both

Na^+ and Cl^- to 33% of normal (mean ± S.D., 0.301 ± 0.022; $P < 0.005$). Thus, reductions in extracellular [Cl^-] do not appear to be involved in the increase in V(t) seen with reductions in NaCl in the bathing solution.

To rule out an effect of sucrose on V(t) and to corroborate the results of the anion substitution experiments, Na^+ was replaced isosmotically with the impermeant organic cation N-methylglucamine. In 6 of 6 experiments, reduction of NaCl to 33% of normal levels resulted in a similar increase in V(t) whether sucrose or N-methylglucamine chloride was used to maintain osmolality. This can be clearly seen in Figure 2-3A, which shows data from a single experiment in which NaCl was reduced to 33% of the normal level, with isosmolal substitution by either sucrose (open triangles) or N-methylglucamine chloride (inverted open triangles). Substitution for Na^+ by either of these compounds produced virtually equivalent increases in V(t), which became evident early during the conditioning train and appeared to reach a steady state by the end of the train. These results argue that neither reduction in external [Cl^-] nor osmotic substitution with sucrose are responsible for the increase in V(t) seen in solutions containing reduced amounts of NaCl.

Figure 2-3B shows the effect of extracellular [Na^+] reduction on EPP amplitude during the stimulus train in the same preparation. It is obvious that while sucrose substitution caused a marked increase in control EPP amplitude,

FIGURE 2-3. Ionic specificity of the effect of reduced external [Na^+] on V(t) during 10 impulse trains. Data from a representative experiment in which external [Na^+] was reduced to 33% of normal by isosmotic substitution of NaCl with sucrose (open triangles) or N-methylglucamine chloride (inverted open triangles). Data obtained in media containing 100% of normal NaCl are represented by filled circles. All solutions contained 0.5 mM Ca^{2+}. (A) Effect of reduced external [Na^+] on V(t). (B) Effect of reduced external [Na^+] on the amplitude of extracellularly recorded EPPs. EPP amplitudes are normalized to the amplitude of the first EPP of the train collected in 100% Na^+ bathing medium.


A
1.2
1.0
0.8
0.6
0.4
0.2
0.0
V(t)
0
100
200
300
400
TIME (ms)
B
260
220
180
140
100
60
EPP AMPLITUDE
0
100
200
300
400
TIME (ms)

N-methylglucamine substitution produced little or no change in control EPP amplitude. A similar effect on control EPP amplitude was noted in 6 of 6 experiments in which N-methylglucamine was substituted for Na^+ in the bathing medium. These results indicate that an increase in V(t) may be produced relatively independently of an increase in control EPP amplitude (see Discussion). The increase in control EPP amplitude seen in low-[Na^+] solutions containing sucrose may be due to a specific effect of sucrose, as substitution of external [Cl^-] by acetate had little effect on control EPP amplitude (see above).

Time Course and Reversibility of the Effect of Extracellular Sodium Reduction on V(t)

In approximately two-thirds of experiments, an increase in EPP amplitude and V(50 ms) could be detected during the first conditioning train delivered following a reduction in extracellular [Na^+]. The increase in V(50 ms) required 5-10 minutes to reach a maximum. Figure 2-4 shows a plot of V(50 ms) during a single experiment in which the Na^+ concentration of the bathing medium was alternated between 100% and 33% of the control level. In this experiment, paired pulses were delivered to the muscle nerve every 40 s. Reduction of extracellular [Na^+] from 100% to 33% of normal led to an approximate doubling of V(50 ms). The effect of reduced [Na^+] on V(t) could be readily reversed by returning the preparation to the control Ringer's solu-

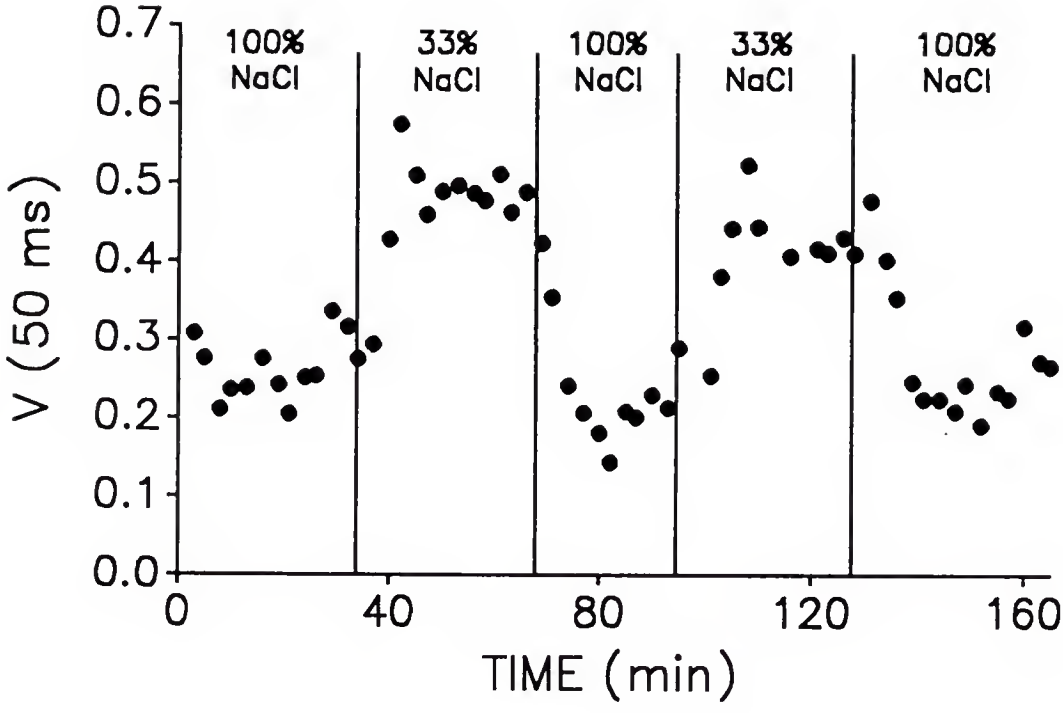


FIGURE 2-4. Time course and reversibility of the effect of reduced external [Na^+] on V(50 ms). Data from a single representative experiment in which paired pulses, 50 ms apart, were delivered to the sartorius muscle nerve every 40 s. All bathing media contained 0.6 mM Ca^{2+}. In 33% NaCl solutions, osmolality was maintained by the addition of sucrose. Each data point represents the average of 4 trials, smoothed using a 3-point moving-bin method as described in Rahamimoff and Yaari (1973), with a delta bin of 1 point. Although in this experiment an initial peak of V(50 ms) was seen shortly after changing from 100% Na^+ to 33% Na^+ solutions, this effect was not consistently observed in other experiments.

tion, and reproduced by a repeated exposure of the preparation to a 33% Na^+ bathing medium.

Concentration Dependence of the Extracellular Sodium Effect on V(t)

The magnitude of the increase in V(50 ms) appeared to be dependent upon the amount of reduction in extracellular $[Na^+]$. This is shown in Figure 2-5, which summarizes data obtained from 27 preparations in 0.5 mM Ca^{2+} bathing solutions in which V(50 ms) was measured at extracellular Na^+ concentrations of 33-100% of normal. Although there was considerable variability in both the control EPP amplitude and the magnitude of the Na^+ effect on V(t) from preparation to preparation, V(50 ms) was clearly increased as extracellular $[Na^+]$ was reduced. The effect of reducing extracellular $[Na^+]$ on V(50 ms) was also measured at extracellular Ca^{2+} concentrations of 0.4 and 0.6 mM (these measurements are later presented in more detail in Figures 2-7 and 2-8). The magnitude of the Na^+ effect on V(50 ms) was clearly dependent on the magnitude of the reduction in external $[Na^+]$ for each concentration of $[Ca^{2+}]_o$ tested.

Effect of Increased Extracellular Sodium on V(t)

To further characterize the concentration dependence of the effect of extracellular sodium on V(t), experiments were performed in which the $[Na^+]$ in the bathing medium was increased above normal levels. However, increasing external $[Na^+]$ also increases the osmolality of the bathing solution. To control for this increase in osmolality,

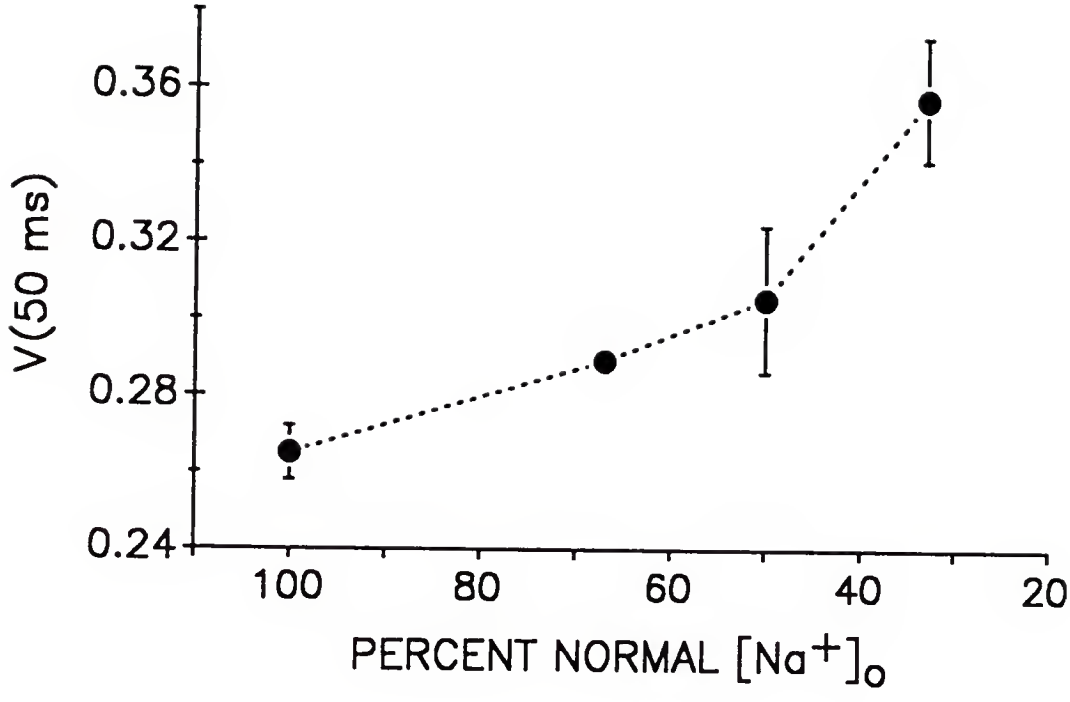


FIGURE 2-5. Dependence of V(50 ms) on external Na^+ concentration. Points represent mean ± S.E.M. of data averaged from experiments performed at an external Ca^{2+} concentration of 0.5 mM, using 10 impulse conditioning trains. In solutions containing reduced Na^+, osmolality was maintained by the addition of sucrose. The following numbers of experiments were averaged for each data point: 100% Na^+, 27; 50% Na^+, 7; 33% Na^+, 18. The data point for V(50 ms) at 67% Na^+ represents a single experiment. Differences between means are statistically significant for the 100% and 50% Na^+ data ($P < 0.01$), the 50% and 33% Na^+ data ($P < 0.05$), and the 100% and 33% Na^+ data ($P < 0.005$).

sucrose was added to solutions containing normal [Na^+]. In 100% Na^+ media, an increase in osmolality caused an increase in control EPP amplitude and a marked reduction in V(t) which was most pronounced near the end of the train (data not shown). In solutions of equivalent osmolality, increases in extracellular [Na^+] cause a reduction in V(t) which is obvious early in the course of the train, and appears to reach a steady state by the end of the train. This can be more clearly seen in Figure 2-6, which summarizes data obtained from 6 preparations exposed to 100% Na^+ bathing solutions (osmolality adjusted with sucrose; filled squares) and 150% Na^+ bathing solutions (open squares). An easily reversible reduction in V(t) was noted in all six preparations upon exposure to bathing solutions containing 150% of the normal Na^+ concentration.

The magnitude of the reduction in V(t) due to increased external [Na^+] appeared to be concentration-dependent. This can be more clearly seen in Table 2-1, which summarizes the effects of increased external [Na^+] on V(50 ms) in 6 experiments in which $[Na^+]_o$ was increased to 125% of normal and in 6 experiments (also depicted in Figure 2-6) in which $[Na^+]_o$ was increased to 150% of normal. Increasing $[Na^+]_o$ to 125% of normal resulted in an average reduction of 9.2% in V(50 ms), while increasing $[Na^+]_o$ to 150% of normal resulted in an average reduction in V(50 ms) of 19%. The results described above suggest that the concentration dependence of the Na^+ effect on V(t) extends to

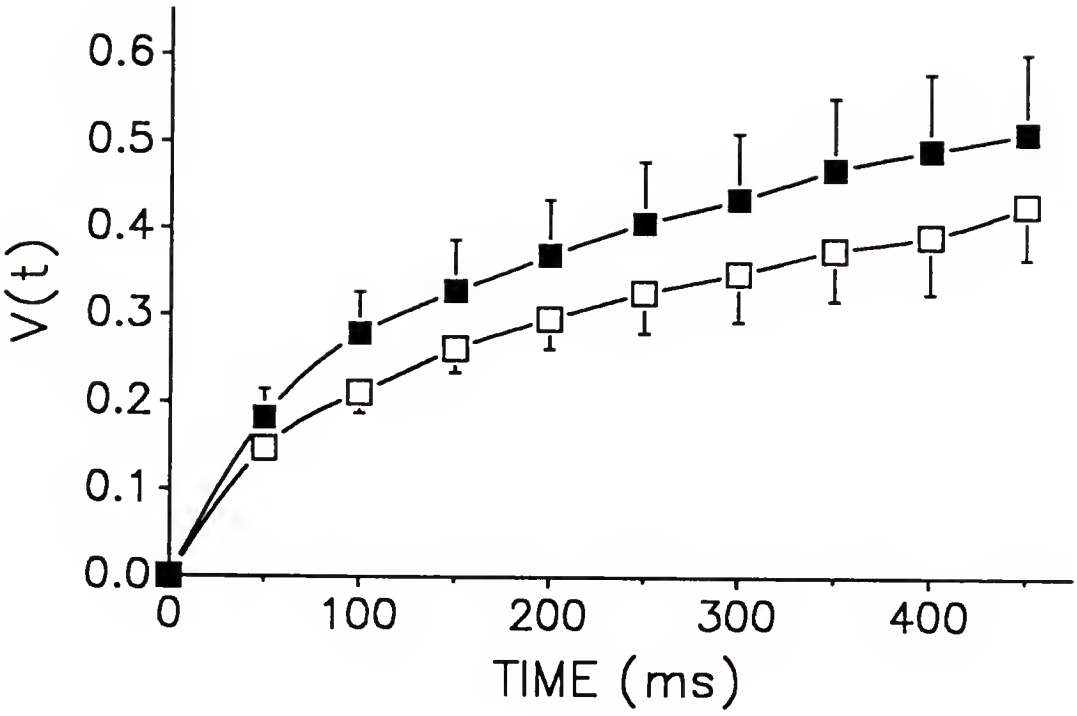


FIGURE 2-6. Effect of increased external [Na^+] on V(t) during 10 impulse conditioning trains. Data points represent mean ± S.E.M. of V(t) measured in 6 experiments conducted at an external [Ca^{2+}] of 0.5 mM. Bathing solutions contained either 150% of normal NaCl (open squares) or 100% of normal NaCl, with osmolality increased to 150% of normal by the addition of sucrose (filled squares). The difference between the population distributions of the V(50 ms) measurements in 100% and 150% NaCl bathing solutions is statistically significant (Wilcoxon rank-sum test for paired experiments, $P < 0.025$).

TABLE 2-1. EFFECT OF INCREASES IN EXTERNAL [Na^+] ON V(50 ms)

[Na^+] (% normal)	Osmolality (% normal)	V(50 ms) (mean ± S.D.)
100[a]	125	0.229 ± 0.031
125	125	0.208 ± 0.020[b]
100[a]	150	0.182 ± 0.074
150	150	0.147 ± 0.022[c]

[a]Osmolality increased to that of the corresponding high-Na^+ solution by the addition of sucrose.
[b]Difference from 100% Na^+, 125% normal osmolality data is statistically significant (Wilcoxon rank-sum test for paired experiments, $P = 0.05$).
[c]Difference from 100% Na^+, 150% normal osmolality data is statistically significant (Wilcoxon rank-sum test for paired experiments, $P < 0.025$).

conditions in which the external $[Na^+]$ has been increased above normal concentrations. However, the magnitude of the change in V(t) for a given increase in $[Na^+]_o$ is smaller than that seen with a similar reduction in extracellular $[Na^+]$.

Effect of Extracellular Calcium Concentration on the Magnitude of the Increase in V(t) Due to Reduced Extracellular Sodium

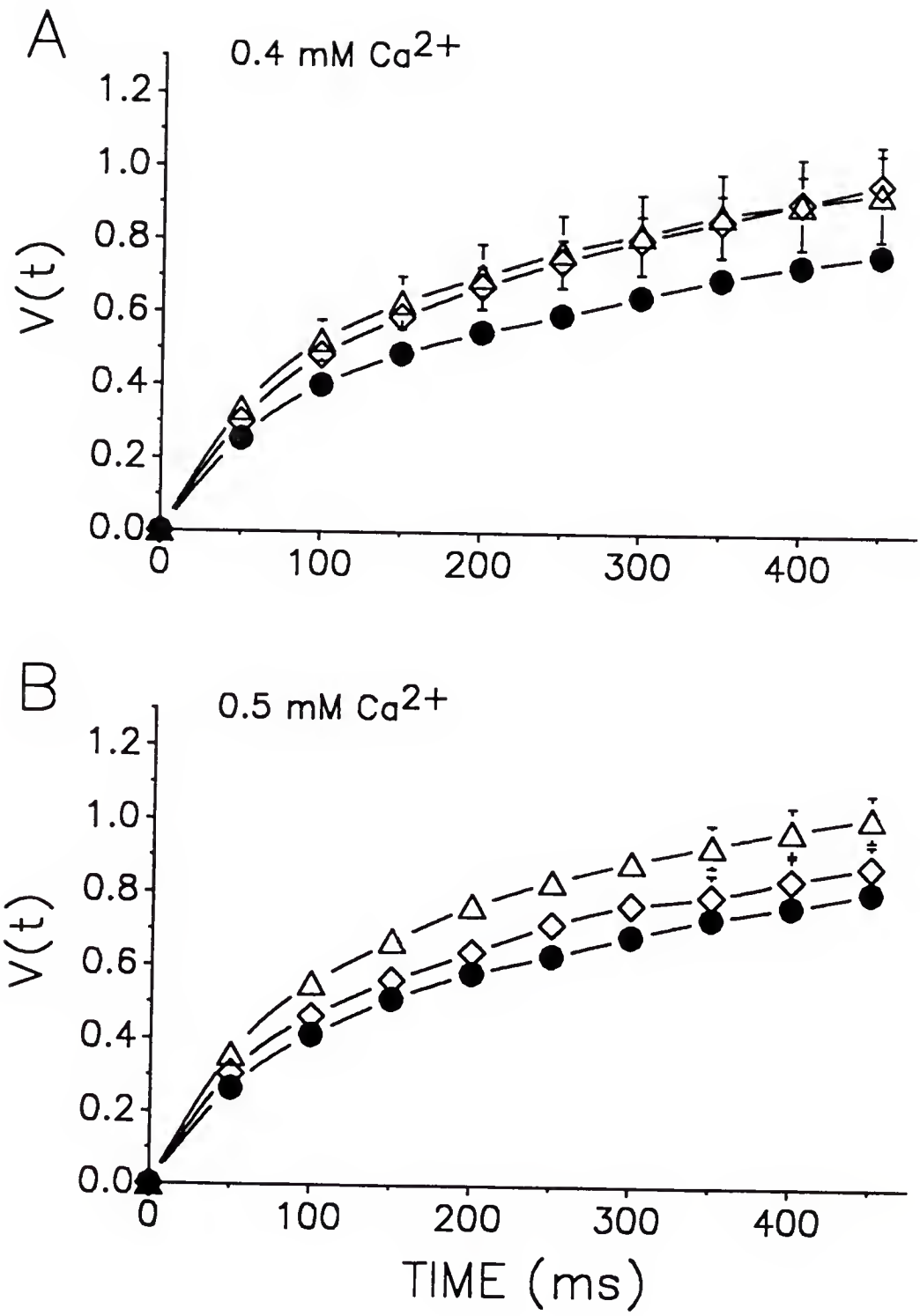
Small elevations of extracellular $[Ca^{2+}]$ under low quantal content conditions have been shown in a series of related experiments performed in this laboratory to increase V(t) late in the course of 10 impulse conditioning trains (Mosier et al., 1986; see Discussion). This effect of raised extracellular $[Ca^{2+}]$ on V(t) appears to be distinguishable in its time course from the effect of reduced extracellular $[Na^+]$ on V(t). Comparison of the Ca^{2+} dependence of the Na^+ effect on V(t) with the direct effect of increased extracellular $[Ca^{2+}]$ on V(t) could provide additional evidence as to whether these two effects represent distinguishable processes underlying increased release. Furthermore, a demonstrable Ca^{2+} dependence of the Na^+ effect on V(t) would be consistent with the hypothesis that changes in extracellular Na^+ may alter release by affecting levels of Ca^{2+} or a Ca^{2+}-activated factor within the nerve terminal.

It was possible to measure the magnitude of the sodium effect on V(t) at extracellular Ca^{2+} concentrations from 0.4 to 0.6 mM. At higher Ca^{2+} concentrations, EPPs often

evoked action potentials in the muscle during a conditioning train; at lower Ca^{2+} concentrations, EPP amplitudes were often so small and variable as to preclude accurate estimates of V(t). As is shown in Figure 2-7, the effect of reduced extracellular $[Na^+]$ on V(t) is increased at higher levels of extracellular $[Ca^{2+}]$. At all concentrations of extracellular Ca^{2+}, the increase in V(t) is manifested early during the conditioning train, and appears to reach a steady state before the end of the train, similar to the time course of the Na^+ effect depicted in Figure 2-2B.

The Ca^{2+} dependence of the Na^+ effect is more clearly shown in Figure 2-8, which plots the dependence of V(50 ms) on extracellular $[Na^+]$ at three different calcium concentrations. In this figure, which shows data averaged from a number of experiments, the ordinate represents the increase in V(50 ms) at a given extracellular Na^+ concentration over the value of V(50 ms) measured at normal Na^+ concentrations. The magnitude of the extracellular $[Na^+]$ effect on V(50 ms) was virtually the same in bathing media containing 0.4 and 0.5 mM Ca^{2+}. Increasing extracellular $[Ca^{2+}]$ to 0.6 mM causes a marked increase in the magnitude of the Na^+ effect on V(50 ms). This effect of increased extracellular $[Ca^{2+}]$ cannot easily be explained by a direct effect of Ca^{2+} on V(t), as measurements of V(50 ms) differed by less than 10% among the three Ca^{2+} concentrations tested at normal Na^+ concentrations.

FIGURE 2-7. Magnitude of the effect of reduced external $[Na^+]$ on V(t) at different concentrations of external Ca^{2+}. Data represent mean $\pm$ S.E.M. Osmolality in all reduced-$[Na^+]$ solutions was maintained by the addition of sucrose. Solutions contained 100% (filled circles), 50% (open diamonds), or 33% (open triangles) of normal $[Na^+]$. (A) Data obtained in 0.4 mM Ca^{2+} bathing solutions. Data were averaged from 14 experiments at 100% Na^+, 7 experiments at 50% Na^+, and 6 experiments at 33% Na^+. Differences between means of V(50 ms) data were statistically significant for the 100% and 50% Na^+ data ($P < 0.025$), and for the 100% and 33% Na^+ data ($P < 0.005$), but not for the 50% and 33% Na^+ data ($P > 0.10$). (B) Data obtained in 0.5 mM Ca^{2+} bathing solutions. Data were averaged from 27 experiments at 100% Na^+, 7 experiments at 50% Na^+, and 18 experiments at 33% Na^+. Differences between means of V(50 ms) values were statistically significant for the 100% and 50% Na^+ data ($P < 0.01$), for the 50% and 33% Na^+ data ($P < 0.05$), and for the 100% and 33% Na^+ data ($P < 0.005$). (C) Data obtained in solutions containing 0.6 mM Ca^{2+}. Data were averaged from 17 experiments at 100% Na^+, 7 experiments at 50% Na^+, and 9 experiments at 33% Na^+. Differences between means of V(50 ms) values were statistically significant for the 100% and 50% Na^+ data ($P < 0.005$), for the 50% and 33% Na^+ data ($P < 0.05$), and for the 100% and 33% Na^+ data ($P < 0.005$).


A
0.4 mM Ca2+
V(t)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
B
0.5 mM Ca2+
V(t)
1.2
1.0
0.8
0.6
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TIME (ms)

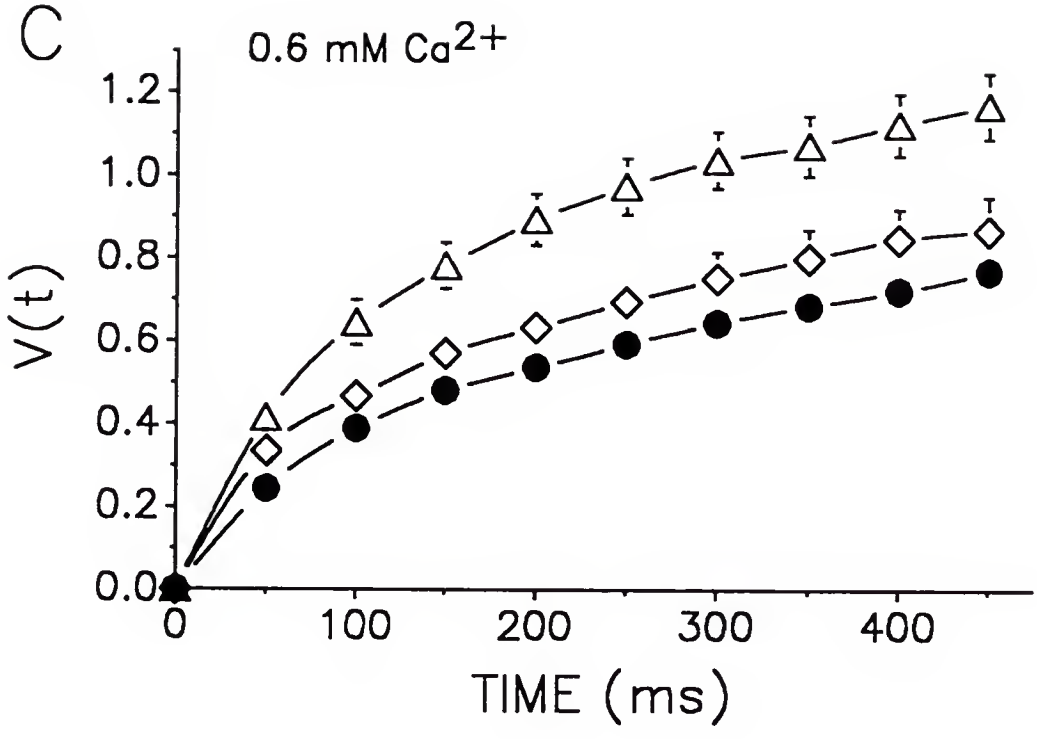


FIGURE 2-7 continued

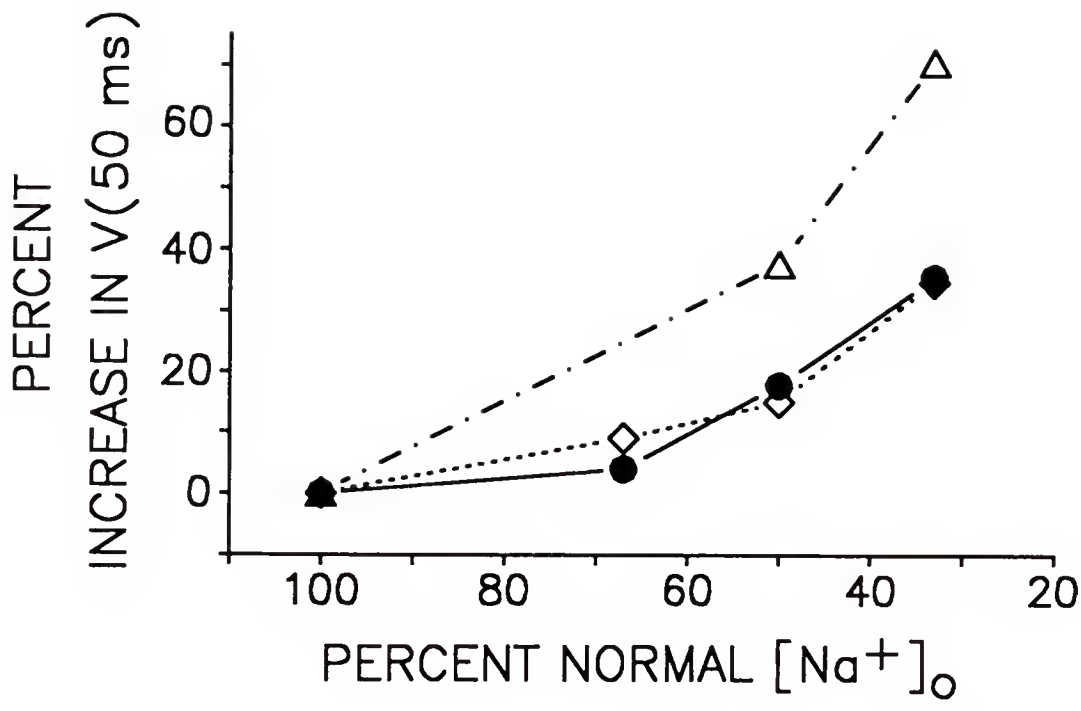


FIGURE 2-8. Effect of external $[Ca^{2+}]$ on the magnitude of the low-$[Na^{+}]$ effect on V(50 ms). Values of V(50 ms) were obtained from the same data as Figure 2-7. Points represent the percent increase of V(50 ms) over the value of V(50 ms) recorded at 100% of normal $[Na^{+}]$, for each concentration of external Ca^{2+}. Data were obtained at external Ca^{2+} concentrations of 0.4 (filled circles), 0.5 (open diamonds), and 0.6 mM Ca^{2+} (open triangles).

Lithium Partially Replaces Sodium in Maintaining V(t)

Lithium ions can substitute for sodium ions in a number of sodium-dependent enzymes and transport mechanisms, whereas lithium is a competitive antagonist of sodium in other systems. Replacement of varying amounts (10-50%) of extracellular sodium with lithium ions had only a small effect on extracellularly recorded V(t). Figure 2-9 summarizes data collected from 6 experiments in which 50% of the Na^+ in the bathing medium was replaced with Li^+. In these experiments, V(50 ms) measured in solutions containing 50% Na^+/50% Li^+ was slightly higher than in control (100% Na^+) bathing solutions (mean $\pm$ S.D., 0.178 $\pm$ 0.023, 100% Na^+; 0.203 $\pm$ 0.021, 50% Na^+/50% Li^+; $P < 0.05$). Reduction of extracellular [Na^+] to 50% of normal, with osmolality maintained by the addition of sucrose, resulted in a larger increase in V(50 ms) over control (0.228 $\pm$ 0.044; $P < 0.025$). These results suggest that lithium ions can substitute for sodium ions in the mechanism underlying early changes in V(t), albeit at somewhat reduced efficiency.

Intracellular Measurements of the Effect of Reduced Extracellular Sodium on V(t)

Intracellular experiments were performed to confirm that the observed increments in V(t) were presynaptic in nature. Quantal content was estimated as described in the Materials and Methods. In addition, MEPP amplitude and resting MEPP frequency were measured directly from the

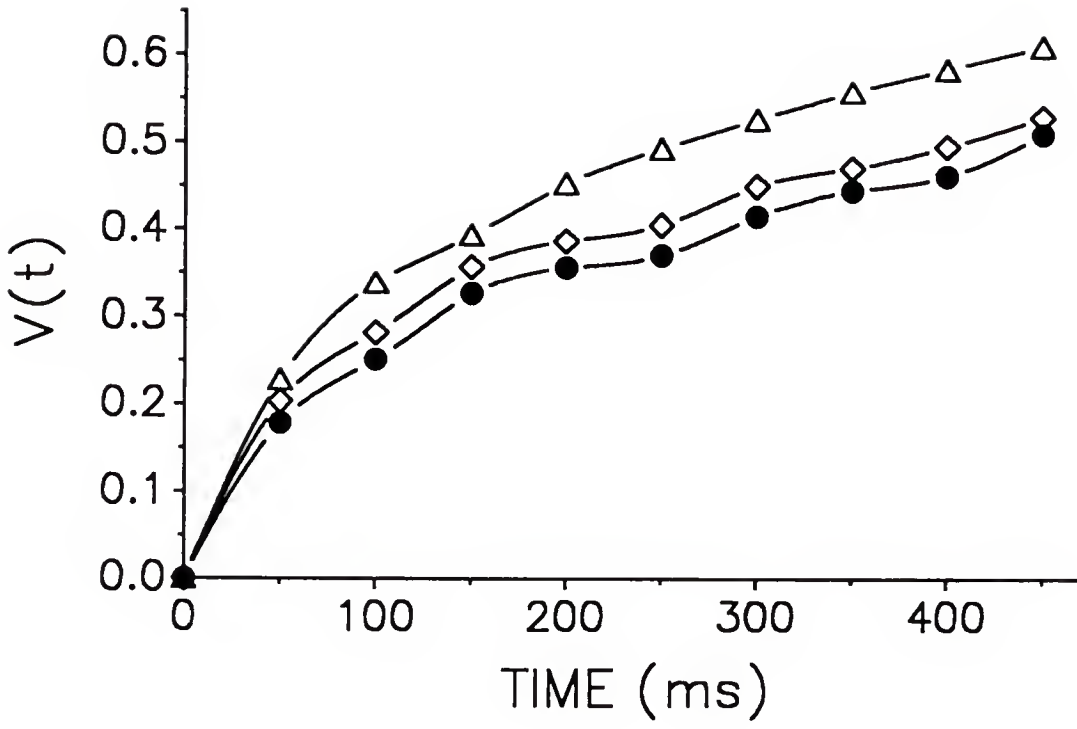


FIGURE 2-9. The effect on V(t) of partial replacement of sodium with lithium. Data averaged from 6 preparations in bathing solutions containing 0.4 mM Ca^{2+}. Bathing solutions were alternated between media containing normal Na^{+} concentrations (filled circles), 50% of normal [Na^{+}] with isosmotic replacement by Li^{+} (open diamonds), and 50% of normal [NaCl] with osmolality maintained by addition of sucrose (open triangles).

screen of the storage oscilloscope. Reduction of extracellular [Na^+] to 50% of normal had no obvious effect on resting MEPP frequency, but decreased MEPP amplitude (as would be expected, since a large portion of the end-plate current is carried by Na^+; Takeuchi and Takeuchi, 1960). This decrease in MEPP amplitude, together with the increase in quantal content observed with decreases in $[Na^+]_o$ (Birks and Cohen, 1965, 1968b), made accurate measurements of quantal content extremely difficult in low-Na^+ solutions. As a result, in only 3 of 20 intracellular experiments could the effect of reduced external [Na^+] on V(t) be characterized (see Materials and Methods).

Figure 2-10 depicts the stimulation-induced increase in both quantal content and intracellularly recorded EPP amplitude in a representative experiment during 10 impulse trains. Only a slight increase in quantal content (filled circles) and EPP amplitude (open circles) was noted during the 10 impulse train in solutions containing 100% of normal [Na^+]. However, in 50% Na^+ solutions, a clear increase in both quantal content (filled triangles) and EPP amplitude (open triangles) was noted during the train. Under both experimental conditions, increases in EPP amplitude are paralleled by increases in quantal content, confirming that changes in V(t) represent changes in transmitter release. Therefore, the increase in V(t) due to reduction of external [Na^+] can be accounted for by an increase in transmitter release. As was noted in the extracellularly recorded

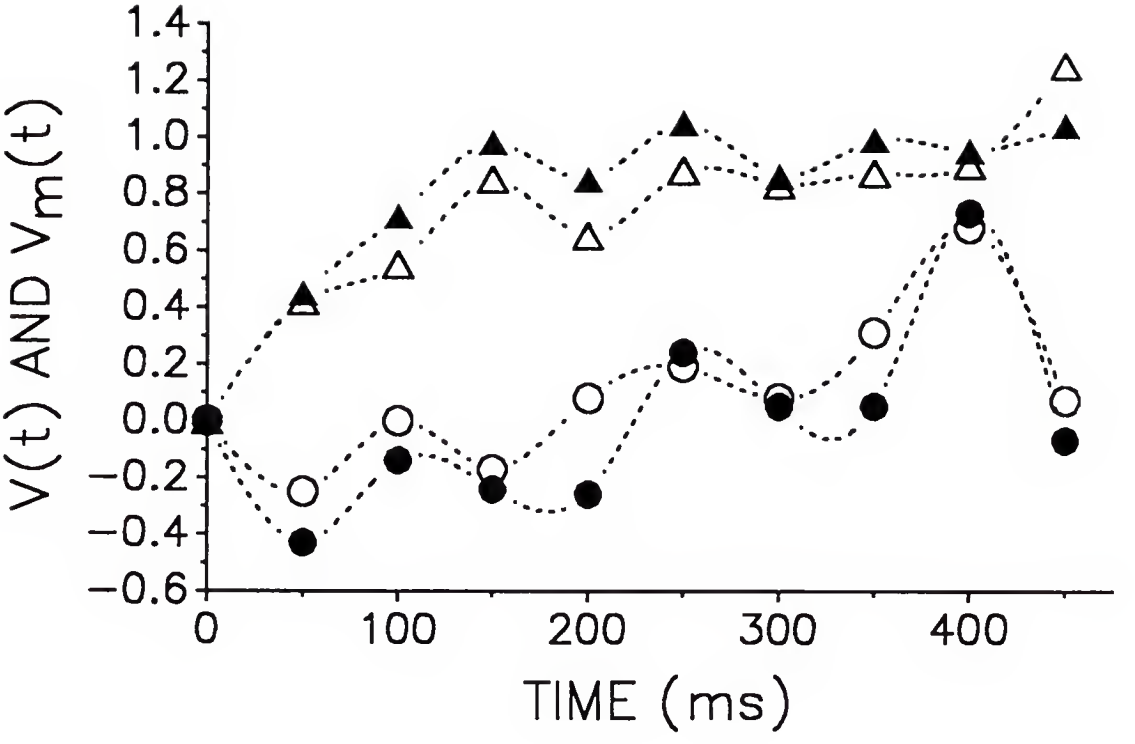


FIGURE 2-10. Intracellular measurements of the effect of reduced external [Na^+] on changes in quantal content during 10 impulse trains. Ordinates represent $V_m(t)$, the change in the quantal content with repetitive stimulation, and V(t), the change in the corresponding EPP amplitude with repetitive stimulation. Quantal content was measured by the method of failures and the method of coefficient of variation as described in the Materials and Methods section. Data are from a single representative experiment performed at a bath [Ca^{2+}] of 0.5 mM. The filled circles represent $V_m(t)$ and the open circles represent V(t) in a 100% Na^+ bathing medium. The filled triangles represent $V_m(t)$ and the open triangles represent V(t) in a 50% Na^+ bathing medium, with osmolality maintained by the addition of sucrose.

data, much of the increase in quantal content due to reduced external [Na^+] occurred at the beginning of the stimulus trains.

Effects of Manipulations of Extracellular Sodium on the Presynaptic Action Potential

One way that reduced extracellular [Na^+] could affect V(t) is through a stimulation-dependent change in the nerve terminal action potential. In particular, an increase in the width of the presynaptic action potential, causing a longer duration of depolarization and a consequent increase in Ca^{2+} entry, has been proposed to underlie increases in transmitter release in Aplysia sensory neurons (Klein and Kandel, 1978). In a small number of experiments, due to a fortuitous placement of the extracellular recording electrode, it was possible to record a presynaptic action potential as well as the end-plate potential. Thus it was feasible in these experiments to measure the width and peak-to-peak amplitude of the extracellularly recorded presynaptic action potential to determine if changes in V(t) could be explained by changes in the shape of the presynaptic action potential.

Figure 2-11 plots the change in action potential width during 10 impulse conditioning trains in solutions containing 100% of normal [Na^+] (filled circles) and in solutions containing 33% of normal [Na^+] with osmolality maintained by sucrose (open triangles) or N-methylglucamine (inverted open triangles). There was little or no difference between

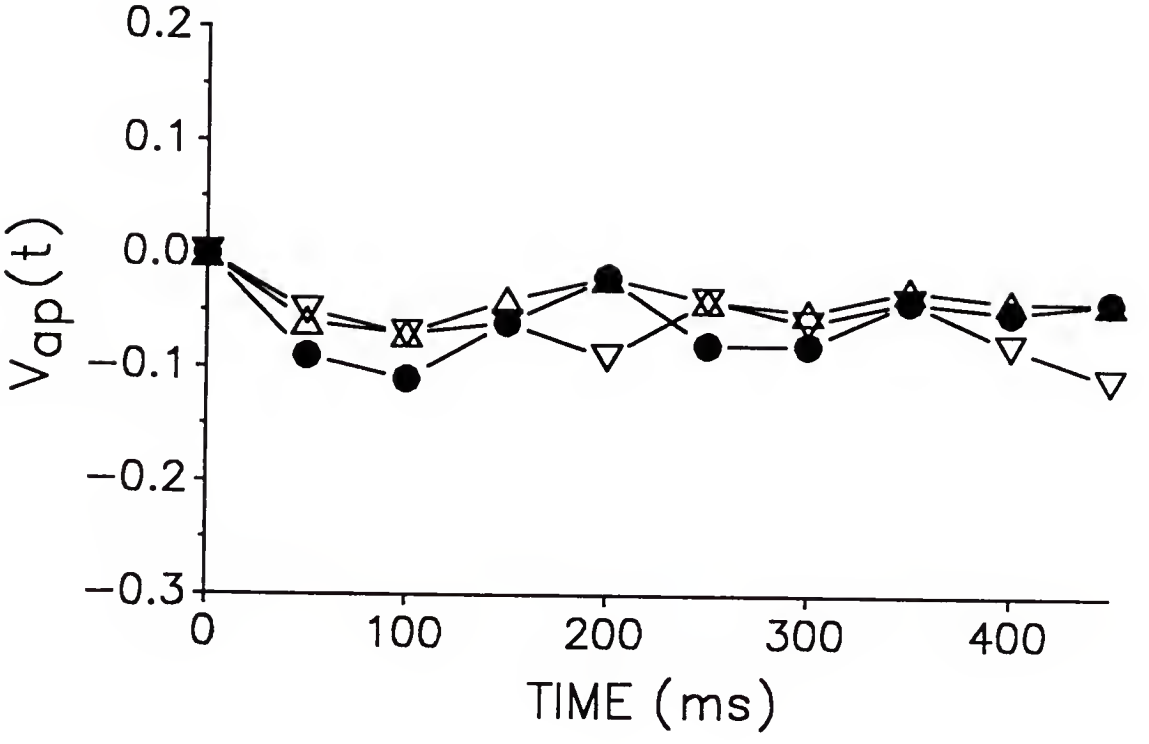


FIGURE 2-11. Effect of reduced external [Na^+] on the change in presynaptic action potential width during repetitive stimulation. Nerve terminal potentials were recorded using a surface electrode. The ordinate represents the ratio of the width of a given action potential to the width of the first action potential in each train. Data are averaged from a number of preparations, all at bath Ca^{2+} concentrations of 0.5 mM. The Na^+ concentration of the bathing solution was varied between 100% of normal (filled circles, n = 10), 33% of normal with osmolality maintained by sucrose (open triangles, n = 9), and 33% of normal with osmolality maintained by N-methylglucamine chloride (inverted open triangles, n = 4).

the stimulation-induced changes in action potential width recorded in 100% Na^+ or 33% Na^+ bathing media. In these same experiments, the average V(50 ms) increased from 0.274 to 0.358 in 100% and 33% Na^+ media, respectively. Likewise, no obvious differences in the amplitude of the action potential were noted with repetitive stimulation in 100% or 33% Na^+ solutions (data not shown), although a small but statistically insignificant decrease in action potential amplitude was noted during the conditioning train under both conditions. These findings are consistent with the findings of previous workers (Hubbard and Schmidt, 1963; Katz and Miledi, 1965; Lev-Tov and Rahamimoff, 1980), who also demonstrated small decreases in the amplitude of extracellularly recorded presynaptic action potentials with repetitive stimulation. The above results suggest that increases in V(t) due to reduction of external $[Na^+]$ cannot be accounted for by stimulation-induced changes in action potential width or amplitude.

As expected, reduction of extracellular $[Na^+]$ to 33% of normal caused a slight reduction in the amplitude of the control action potential (the first action potential of the conditioning train) and an increase in the width of the control action potential in 9 experiments. With prolonged exposure of the preparation to reduced extracellular Na^+, there was a progressive increase in the width of the control action potential, but no additional increase in V(t) beyond that seen immediately after the solution change.

Similar effects of reduced extracellular [Na^+] on action potential width and amplitude were seen in 4 preparations in which N-methylglucamine rather than sucrose was used to maintain osmolality, even though these two methods of Na^+ substitution are markedly different in their effects on control EPP amplitude. In a single experiment, the extracellular action potential was measured during reduction of NaCl to 33% of normal, with isosmotic substitution by lithium chloride and sucrose. In this experiment, a similar increase in action potential width was seen in the presence of low-Na^+ solutions substituted with either sucrose or Li^+, although Li^+ substitution caused little or no increase in either control EPP amplitude or V(t). These experiments suggest that the changes in control action potential width or amplitude seen in the presence of reduced $[Na^+]_o$ have little or no effect on either control EPP amplitude or V(t).

The effect of elevated extracellular [Na^+] on the presynaptic action potential was also examined. No changes in action potential amplitude or width with repetitive stimulation were apparent in 3 experiments in which $[Na^+]_o$ was alternated between 100% and 150% of normal. In these experiments, osmolality was held constant at 150% of normal. Likewise, there was little or no effect of raised $[Na^+]_o$ on control action potential amplitude or width (data not shown). These experiments provide additional evidence that changes in the presynaptic action potential play

little or no role in the changes in V(t) seen with alterations of the extracellular Na^+ concentration.

Effect of Monensin on V(t)

The experiments described thus far cannot distinguish between an effect on V(t) due to a change in internal or external $[Na^+]$ or an effect resulting from a change in the driving force for Na^+ entry into the nerve terminal. Increasing the concentration of intracellular Na^+ offers a way to distinguish between these possibilities by reducing the driving force for Na^+ entry without affecting the external Na^+ concentration. If the effect of reduced external $[Na^+]$ on V(t) is mediated by a corresponding decrease in $[Na^+]_i$ (Thomas, 1972; Deitmer and Schlue, 1983), then an increase in internal $[Na^+]$ should have the opposite effect on V(t) as a reduction in external $[Na^+]$. However, if the effect of reduced $[Na^+]_o$ on V(t) is mediated by a reduction in the driving force for Na^+ across the cell membrane, then an increase in internal $[Na^+]$ should have the same effect on V(t) as a decrease in external $[Na^+]$. One way to examine the effect of increased intracellular $[Na^+]$ on stimulation-increased release is the addition of monensin, a sodium ionophore which inserts into the plasma membrane and acts as a carrier to transport Na^+ across the plasma membrane (Pressman and Fahim, 1982).

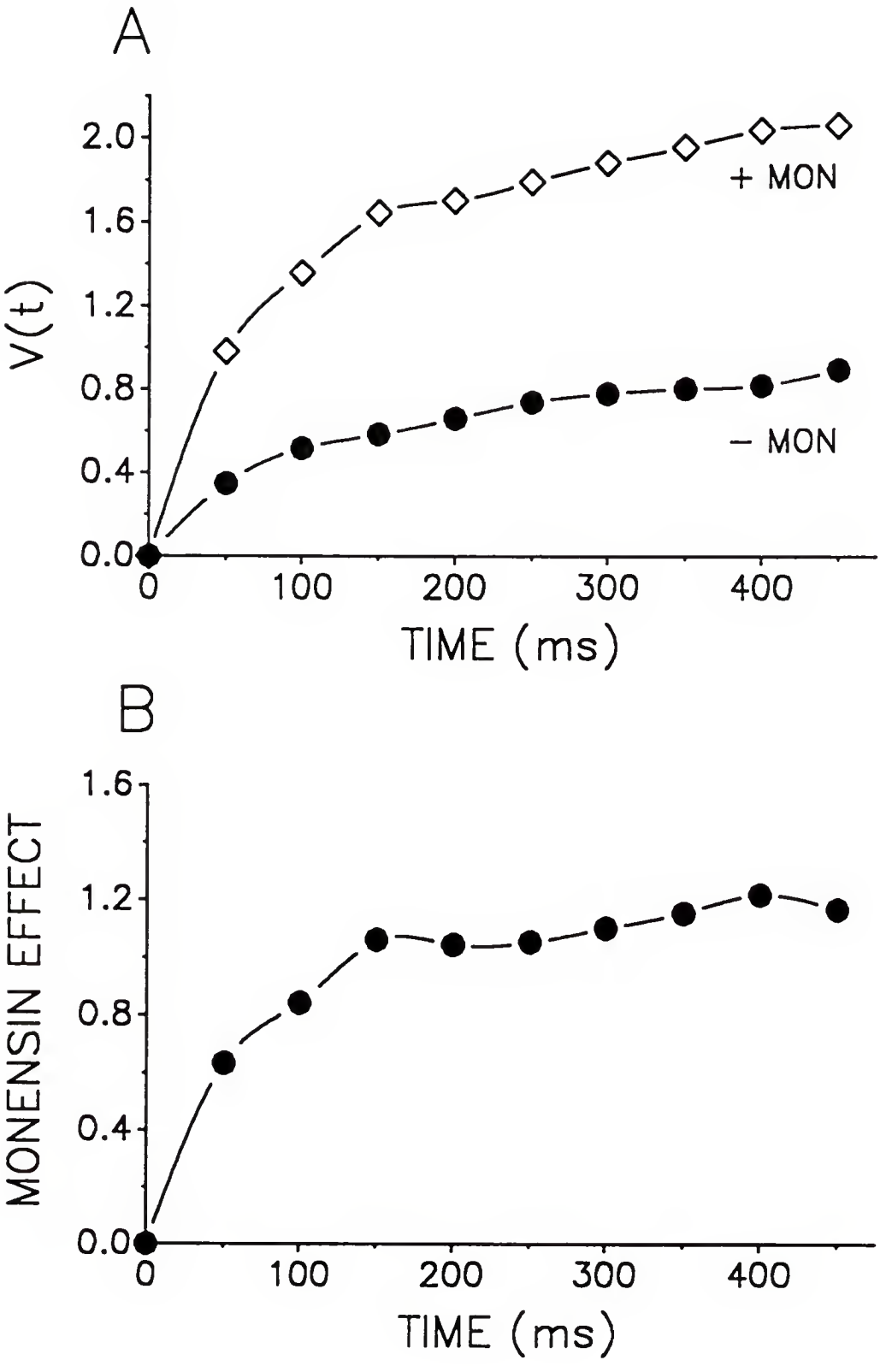
Addition of monensin (dissolved in ethanol) to the bathing medium had no consistent effect on control EPP amplitudes recorded extracellularly in 17 experiments (7

increased; 6 decreased; 4 little or no effect). However, in 13 of the 17 experiments, a striking increase in V(50 ms) was noted upon addition of 0.5-10 uM monensin dissolved in ethanol. In the remaining 4 experiments, addition of monensin had little or no effect on V(t). A similar concentration of ethanol alone (0.8%, v/v) had no discernible effect on control EPP amplitude or V(t) in 5 experiments. Results of a representative experiment are shown in Figure 2-12A. In this experiment, the magnitude of V(50 ms) is 0.35 in a normal [Na^+] bathing solution, and is increased to 0.98 after the addition of 2 uM monensin to the bathing solution. Similar to the effect of reduced extracellular [Na^+] depicted in Fig. 2-1B, most of the monensin-induced increase in V(t) occurred early during the conditioning train, and reached a plateau by the end of the train (Figure 2-12B). The effect of monensin on V(t) usually took 8-20 min to reach stable levels, although an effect was sometimes noticeable during the first two or three trains following its addition. The effect of monensin on V(t) could be reversed readily by washing several times with the control Ringer's solution, and could be reproduced upon re-addition of monensin to the bathing solution.

Effect of Ouabain on V(t)

Inhibition of the Na^+/K^+ pump with cardiac glycosides has been shown to result in accumulation of intracellular Na^+ in a number of preparations (Thomas, 1972; Ellis and

FIGURE 2-12. Effect of the Na^+ ionophore monensin on V(t) during 10 impulse trains. (A) Data from a representative experiment performed at a bath [Ca^{2+}] of 0.5 mM. The bathing medium was alternated between a control solution (filled circles) and a solution containing 2 uM monensin (open diamonds). (B) Time course of the increase in V(t) attributable to monensin. Data are from the same experiment as (A), representing the difference between measures of V(t) with and without monensin in the bathing medium.


A
2.0
1.6
1.2
0.8
0.4
0.0
V(t)
+ MON
– MON
0
100
200
300
400
TIME (ms)
B
1.6
1.2
0.8
0.4
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MONENSIN EFFECT
0
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400
TIME (ms)

Deitmer, 1978; Deitmer and Schlue, 1983). As Na^+/K^+ pump inhibition offers an alternative method of increasing $[Na^+]_i$ at the frog neuromuscular junction, experiments were performed to ascertain the effect of the cardiac glycoside ouabain on increased release during 10 impulse conditioning trains.

Surprisingly, addition of 2.8-20 uM ouabain to the bathing medium had little or no effect on V(t) during 10 impulse trains in 8 of 10 experiments, although V(t) appeared to increase in 1 experiment and decrease in 1 experiment. After a 15-60 minute delay, ouabain also led to a rapid increase in EPP amplitude, similar to the findings of Birks and Cohen (1968a). More prolonged (35-90 min) exposure to ouabain resulted in the sudden onset of failure of neuromuscular transmission. This is probably a result of failure of impulse conduction in the nerve to the sartorius muscle, as has been described previously (Birks and Cohen, 1968a,b; Baker and Crawford, 1975).

The apparent lack of effect of ouabain on V(t) may be due to the effects of other factors which may have obscured any effect of increased $[Na^+]_i$ on V(t). Inhibition of the Na^+/K^+ pump not only leads to an increase in internal $[Na^+]$ but also to a reduction of internal $[K^+]$, with concomitant membrane depolarization (e.g., Banks, 1967). The latter effects of Na^+/K^+ pump inhibition may affect transmitter release independently of changes in external or internal Na^+. Furthermore, the striking increase in quantal release

observed with ouabain treatment may have led to a depletion of quanta available for release, causing depression of transmitter release during conditioning trains (e.g., Thies, 1965). Finally, measurements of V(t) during ouabain exposure were only feasible in the interval preceding the phase of rapid increases in control EPP amplitude, when the accumulation of internal Na^+ may have been insufficient to affect the driving force for Na^+ entry. Further experiments employing alternative methods of increasing internal $[Na^+]$, such as the introduction of Na^+ into the nerve terminal with liposomes (e.g., Rahamimoff et al., 1978), would be desirable in order to clarify these difficulties.

Effect of Amiloride on V(t)

The effects on V(t) of reduced $[Na^+]_o$, which leads to reduced $[Na^+]_i$, and monensin, which increases $[Na^+]_i$, are qualitatively similar, suggesting that a mechanism dependent upon the transmembrane Na^+ gradient may underlie the expression of V(t) during 10 impulse trains (see Discussion). One mechanism which may depend upon the transmembrane Na^+ gradient for its operation is the Na^+/H^+ exchanger, a carrier protein which regulates intracellular pH by Na^+-gradient-driven removal of H^+ from the cytosol. Intracellular pH changes are known to affect cytosolic Ca^{2+} handling and a number of membrane electrical properties in excitable cells (reviewed in Moody, 1984), and could conceivably play a role in the expression of stimulation-induced increases in release.

To determine whether a Na^+/H^+ exchange mechanism is involved in the expression of V(t) during short conditioning trains, a number of preparations were exposed to the Na^+/H^+ exchange inhibitor amiloride. Since amiloride has been reported to have little or no effect on Na^+/Ca^{2+} exchange in squid axons (Allen and Baker, 1986), the use of amiloride offers a way to distinguish between the effects on V(t) of Na^+/H^+ exchange and Na^+/Ca^{2+} exchange. The amiloride concentration required to achieve half-maximal inhibition of the Na^+/H^+ exchanger has been reported to be in the range of 2-5 uM in a number of non-renal cells (Frelin et al., 1987). As can be seen in Figure 2-13, which presents averaged data from 6 preparations, exposure of the frog sartorius nerve-muscle preparation to amiloride concentrations as high as 50 or 500 uM resulted in little or no change in V(t) during 10 impulse conditioning trains. No obvious change in V(t) during exposure to amiloride was seen in any of the individual experiments which were averaged for Figure 2-13. It can be concluded that the Na^+/H^+ exchange mechanism probably plays little role in the expression of the increase in V(t) seen with reductions of extracellular $[Na^+]$.

Discussion

Effect on V(t) of Alterations in Sodium is Distinguishable from the Effect of Altered External Calcium

At least four components of increased transmitter release have been defined at the frog neuromuscular junc-

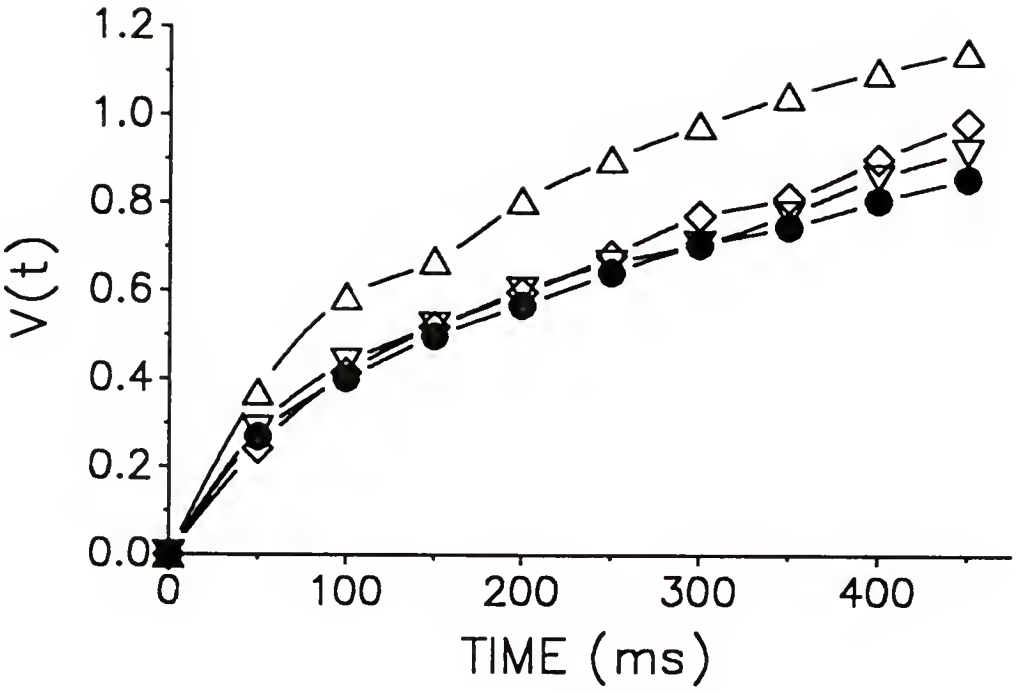
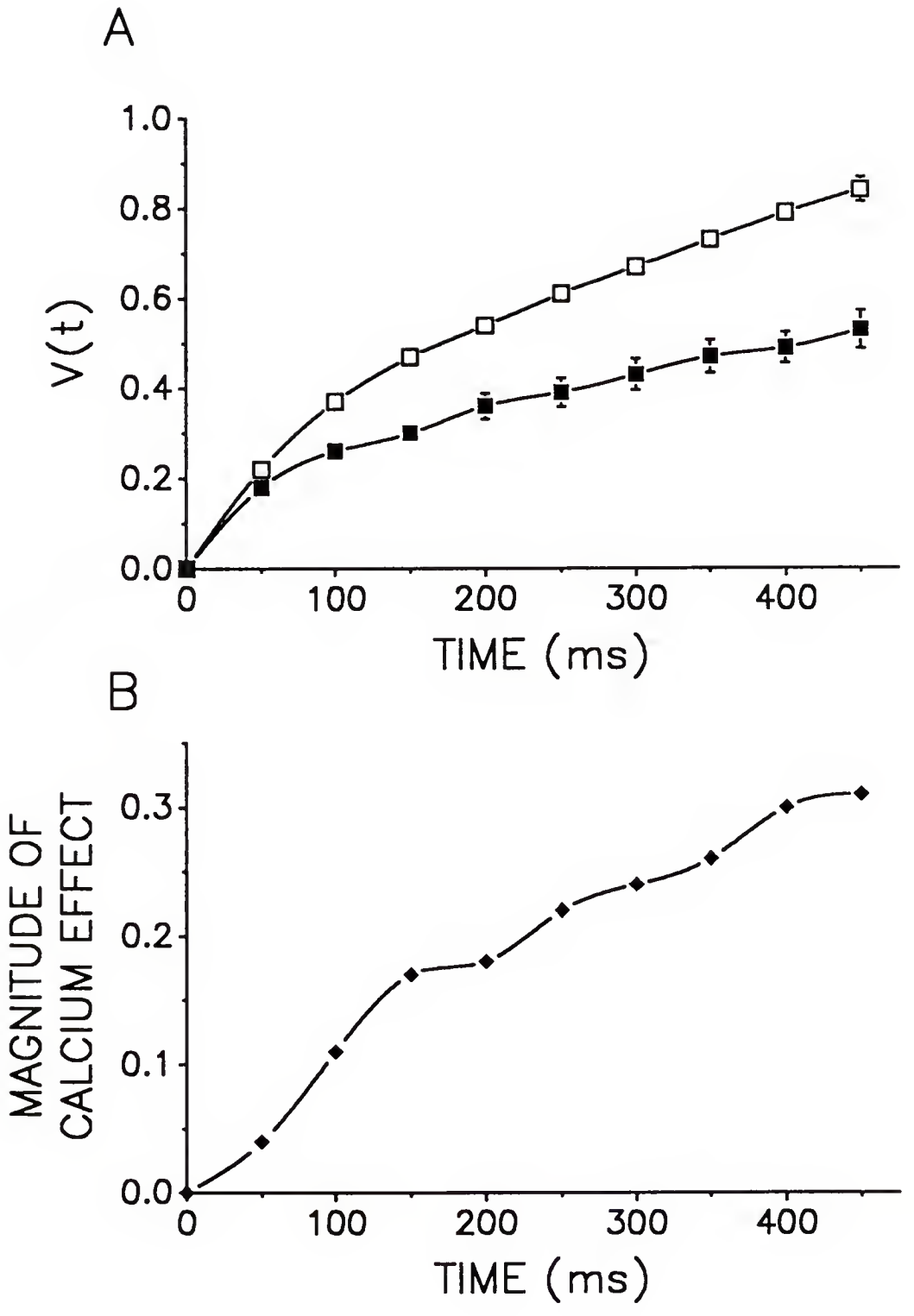


FIGURE 2-13. Effect of the Na^+/H^+-exchange inhibitor amiloride on V(t) during 10 impulse trains. Data are averaged from a number of experiments in which V(t) was measured in solutions containing 0.5 mM Ca^{2+}. Solutions are: normal $[Na^+]$ without amiloride (filled circles, n = 6); normal $[Na^+]$ with 50 uM amiloride added (inverted open triangles, n = 4); normal $[Na^+]$ containing 500 uM amiloride (open diamonds, n = 2); and 33% of normal $[Na^+]$ without amiloride (open triangles, n = 2).

tion. These are believed to reflect four distinct biophysical or biochemical processes which occur within the nerve terminal during repetitive stimulation. While changes in intra-terminal calcium pools are thought to underlie many, if not all, of the components of increased release, sodium ions have also been proposed to play a key role in regulating one or more of these pools. The data presented in this dissertation suggest that the first component of facilitation, a process with a decay constant of 50-60 ms (Zengel and Magleby, 1982), may be selectively affected by manipulations of extracellular and intracellular [Na^+].

Alterations in external [Ca^{2+}] have little effect on V(50 ms) in solutions containing normal concentrations of external Na^+, while they appear to increase a longer-lasting component of increased release (Mosier et al., 1986). The effect of increases in external [Ca^{2+}] on V(t) is demonstrated in Figure 2-14A, which plots averaged V(t) data obtained in this laboratory. As the external [Ca^{2+}] is increased from 0.4 mM (filled squares) to 0.6 mM (open squares), V(t) is increased. However, the increase in V(t) is small early in the course of the 10 impulse train, and reaches its greatest magnitude at the end of the train. This can be seen more clearly in Figure 2-14B, which plots the difference between the V(t) data shown in Figure 2-14A. It is obvious that the change in V(t) attributable to increased external [Ca^{2+}] continues to increase during the

FIGURE 2-14. Effect of increased external [Ca^{2+}] on V(t). (A) Averaged V(t) values from 10 impulse conditioning trains. Data represent mean $\pm$ S.E.M. of V(t) from 15 experiments in media containing 0.4 mM Ca^{2+} (filled squares) and 10 experiments in media containing 0.6 mM Ca^{2+} (open squares). (B) Time course of the increase in V(t) attributable to increased external [Ca^{2+}]. Data are from the same experiments as (A), with ordinates representing the differences between V(t) measured at external Ca^{2+} concentrations of 0.4 and 0.6 mM.


A
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V(t)
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400
TIME (ms)
B
0.3
0.2
0.1
0.0
MAGNITUDE OF
CALCIUM EFFECT
0
100
200
300
400
TIME (ms)

course of the 10 impulse conditioning train, and shows no evidence of approaching a plateau during the train.

On the other hand, changes in external Na^+ exert their principal effect on V(t) early during short conditioning trains delivered to the muscle nerve. The Na^+ effect on V(t) appears to reach a steady state by the end of the 10 impulse train. Comparing Figure 2-2B, which plots the increase in V(t) attributable to reduced external $[Na^+]$, with Figure 2-14B, which plots the increase in V(t) attributable to increased external $[Ca^{2+}]$, shows that the effect of reduced $[Na^+]_o$ on V(t) is markedly distinct from the previously described effect of increased $[Ca^{2+}]_o$ on V(t).

Localization of the Site of Action of Sodium Ions

As all preparations were stimulated at five times the threshold voltage required to produce a maximal control EPP amplitude as recorded by the extracellular electrode, a change in the measured increase in V(t) is unlikely to be due to recruitment of axons. Furthermore, the increase in V(t) due to reduced external $[Na^+]$ could be demonstrated with intracellular as well as extracellular recording techniques, consistent with the hypothesis that the Na^+ effect on V(t) is homosynaptic, i.e., capable of being expressed at a single synapse (see Materials and Methods).

Stimulation-induced changes in the presynaptic action potential are also an unlikely explanation for the observed increases in transmitter release. Facilitation and longer-term components of stimulation-increased transmitter

release are not associated with any consistent changes in the amplitude or width of the presynaptic action potential during repetitive stimulation at a number of crustacean, amphibian, and mammalian synapses (Martin and Pilar, 1964; Ortiz, 1972; Zucker, 1974; Charlton and Bittner, 1978; Zucker and Lara-Estrella, 1979; Baldo et al., 1983). Furthermore, measurements of presynaptic action potential amplitude have actually shown a decrease with repetitive stimulation in some preparations (Katz and Miledi, 1965; Braun and Schmidt, 1966; Zucker, 1974; Lev-Tov and Rahamimoff, 1980), which cannot account for the stimulation-induced increase in EPP amplitude observed. Injection of Ca^{2+} into the presynaptic terminal of the squid giant synapse results in facilitation of transmitter release without a change in spike waveform (Charlton et al., 1982). Facilitation of release can also be demonstrated in synapses in which the action potential has been blocked with tetrodotoxin and the nerve terminal stimulated focally with repetitive impulses of constant amplitude and duration (Zucker, 1974).

Consistent with the aforementioned findings, no stimulation-induced changes in presynaptic action potential amplitude or width which could account for an increase in V(t) were noted in this study. Furthermore, in a number of experiments, marked changes in control action potential amplitude could be detected without a significant change in V(t). Extracellular measurements of the presynaptic action

potential are not sufficiently sensitive to exclude subtle changes in calcium currents occurring at the nerve terminal; however, the lack of a direct effect of changes in extracellular [Ca^{2+}] on the early component of facilitation argues against this possibility.

Postsynaptic changes are also unlikely to contribute to the low-Na^+-induced increase in V(t). Desensitization, the only stimulation-dependent postsynaptic change that has been described at neuromuscular junctions, requires tens of seconds of repetitive stimulation or of continuous exposure to acetylcholine for its expression (e.g., Thesleff, 1955; Magleby and Pallotta, 1981; Ruzzier and Scuka, 1986), and decreases rather than increases the effect of acetylcholine on the postsynaptic membrane. In experiments using intracellular recording techniques, changes in V(t) could be fully explained by changes in quantal release during repetitive stimulation.

The effects of manipulations of [Na^+] on V(t) do not appear to be a function of their effects on control levels of transmitter release. While substitution of NaCl with sucrose often markedly increases both control EPP amplitude and V(t) in this preparation, substitution of NaCl with N-methylglucamine chloride increases V(t) with no significant effect on control EPP amplitude. Monensin, a Na^+ ionophore, dramatically increases V(t) in many preparations with no consistent effect on control EPP amplitude. On the other hand, prolonged exposure to ouabain results in little

or no increase in V(t) while producing a marked increase in control EPP amplitude.

Specificity of the Sodium Effect on V(t)

The results of ion substitution experiments indicate that the increase in V(t) due to NaCl reduction is specific for the sodium ion. Replacement of Cl^- by organic anions such as methylsulfate has been shown to have little effect on muscle membrane potential or MEPP frequency at frog neuromuscular junctions, although replacement with more lyotropic anions such as bromide or nitrate may increase MEPP frequency (Muchnik and Gage, 1968). Substitution of Cl^- by acetate ion has little or no effect on V(50 ms), although it may affect a slower component of increased release. Substitution of Na^+ by N-methylglucamine, an impermeant cation, has a similar effect on V(t) as does substitution of NaCl by sucrose. These results suggest that the increase in V(t) is due to a reduction in external $[Na^+]$ rather than a reduction of Cl^- or a specific effect of sucrose. The results of substitution with Li^+, a permeant cation, indicate that isosmotic substitution with Li^+ can partially but not completely prevent the increase in V(t) which was seen in the presence of reduced external Na^+. This may indicate that Li^+ interacts, albeit somewhat weakly, at the site of action of external Na^+; alternatively, Li^+ may have other actions which could modify V(t), e.g., an effect on the phosphatidylinositol system (reviewed in Downes, 1983).

Possible Mechanisms for the Sodium-Induced Increase in V(t)

A stimulation-dependent increase in Ca^{2+} entry into the nerve terminal could theoretically underlie an increase in V(t); however, evidence supporting such a mechanism is lacking at the frog neuromuscular junction. The amount of Ca influx per impulse into molluscan or leech neurons or squid nerve terminals does not seem to increase during short trains of repetitive stimulation (Smith and Zucker, 1980; Charlton et al., 1982; Ross et al., 1987; but see Barrett and Morita, 1987). In the experiments described above, increases in extracellular Ca^{2+} had little or no direct effect on V(50 ms) during short conditioning trains, although they did seem to increase a longer-lasting component of increased release.

The observed effects of extracellular $[Na^+]$ changes and the Na^+ ionophore monensin are most consistent with a mechanism which depends upon the transmembrane Na^+ gradient of the nerve terminal. Relief of competition between extracellular Na^+ and Ca^{2+} for entry into the nerve terminal, as was first proposed by Colomo and Rahamimoff (1968), could conceivably account for the effect of reduced extracellular $[Na^+]$ on increased release. However, the similar increase in V(t) seen with application of monensin, an ionophore known to increase intracellular $[Na^+]$, is more consistent with a mechanism driven by the transmembrane Na^+ gradient. The effect of monensin, which increases intracellular $[Na^+]$, could possibly be explained by a mechanism

dependent upon the concentration of intracellular Na^+ (reviewed in Rahamimoff et al., 1980). However, the similar effect on V(t) of reduced extracellular [Na^+], which causes a slow decrease in intracellular [Na^+] (Thomas, 1972), cannot be easily explained by this hypothesis. The similar effects on V(t) of manipulations which decrease extracellular [Na^+] or increase intracellular [Na^+] are best explained by a mechanism involving the reduction in the Na^+ gradient across the nerve terminal membrane.

One difficulty with the above interpretation is the seeming lack of effect of ouabain, which should increase intracellular [Na^+], on V(t). However, ouabain may have other effects which act to obscure any increase in V(t) due to elevated intraterminal [Na^+], e.g., synaptic depression due to the marked increase in transmitter release which occurs after a short exposure to ouabain.

If the effect on V(t) of manipulations of extracellular and intracellular Na^+ concentrations is mediated through a mechanism dependent upon the transmembrane Na^+ gradient, what mechanisms might be proposed to underlie this effect? An effect mediated by voltage-dependent sodium channels is unlikely due to the marked dependence of the magnitude of the Na^+ effect on extracellular Ca^{2+}. Intracellular pH changes mediated by Na^+/H^+ exchange have been proposed as a prerequisite for intracellular Ca^{2+} mobilization in other systems (e.g., Siffert and Akkerman, 1987), and decreases in intracellular pH have been measured during trains of

action potentials in molluscan neurons (Ahmed and Connor, 1980). Conceivably a Na^+/H^+ exchanger could affect increased release either directly or indirectly by an alteration in intracellular pH. However, the Na^+/H^+ exchange mechanism is not known to be significantly affected by extracellular $[Ca^{2+}]$ (Grinstein and Cohen, 1987). Furthermore, reduction of external $[Na^+]$ and application of the Na^+ ionophore monensin, which produced qualitatively similar increases in V(t) in this study, are known to have opposite effects on intracellular pH in other systems (acidification and alkalinization, respectively; e.g., Ellis and MacLeod, 1985; Grinstein and Cohen, 1987; Lichtshtein et al., 1979). Finally, application of the Na^+/H^+ exchange inhibitor amiloride had little or no effect on V(t) even at concentrations sufficient to achieve a complete block of the Na^+/H^+ exchanger.

A Na^+/Ca^{2+} exchange mechanism has been proposed to be involved in one or more stimulation-induced changes in transmitter release at vertebrate neuromuscular junctions (Parnas et al., 1982; Meiri et al., 1986; Misler et al., 1987). Na^+/Ca^{2+} exchange is believed to play an important role in the regulation of intracellular Ca^{2+} levels in many biological systems (reviewed in Blaustein, 1988), and a Na^+/Ca^{2+} exchange mechanism has been characterized in preparations of nerve endings from rat brain (Nachshen et al., 1986; Sanchez-Armass and Blaustein, 1987). The Na^+/Ca^{2+} exchanger in rat brain synaptosomes may be able to

extrude Ca^{2+} sufficiently quickly to match Ca^{2+} influx at firing frequencies up to 18/s (Sanchez-Armass and Blaustein, 1987; Blaustein, 1988). Such a rate of Ca^{2+} extrusion would be sufficiently rapid to play a role in even the shortest component of facilitation at the nerve terminal. The partial efficacy of Li^+ in preventing an increase in V(t) due to reduction of external $[Na^+]$ would also be consistent with an effect on a Na^+/Ca^{2+} exchanger, as Li^+ has been shown to reduce the V_{max} of the exchange reaction in purified synaptic plasma membrane vesicles (Hermoni et al., 1987). Such a mechanism would appear to be the most parsimonious explanation for the presumably Na^+ gradient-dependent process characterized in this study, although some other mechanism, as yet uncharacterized, cannot be ruled out.

Conclusion

At least four processes act to increase transmitter release during repetitive stimulation; some or all of these may involve the buildup of residual Ca^{2+} within the nerve terminal between successive impulses. Sodium ions have been proposed to play a role in one or more of these four processes. The data presented in this study support the hypothesis that an early component of increased release, with a time course similar to the previously defined first component of facilitation (Zengel and Magleby, 1982), is selectively affected by manipulations of extracellular Na^+.

The apparent dependence of this process on the transmembrane Na^+ gradient and external Ca^{2+} concentration suggest a Na^+-Ca^{2+} exchange mechanism, although other explanations may be advanced. Further research will be necessary to test this hypothesis, as well as to elucidate the mechanisms underlying the other components of increased release at the neuromuscular junction.

CHAPTER 3

MODELING OF THE EFFECT OF SODIUM ON STIMULATION-INDUCED INCREASES IN RELEASE

Introduction

As previously discussed in Chapter 2, four processes of increased release have been described at the frog neuromuscular junction: two components of facilitation with time constants of decay of approximately 60 and 500 ms, augmentation which decays with a time constant of about 7 s, and potentiation which has a time constant of decay of tens of seconds to minutes (Zengel and Magleby, 1982). It is likely that the different components of increased release reflect different underlying biochemical or biophysical processes which affect transmitter release. Besides marked differences in the kinetic properties of the components of increased release, several findings have added to the evidence that these components are separable and act relatively independently of one another. At frog neuromuscular junctions, externally applied Sr^{2+} and Ba^{2+} ions selectively increase the second component of facilitation and augmentation, respectively, of both evoked release and miniature end-plate potential (MEPP) frequency (Zengel and Magleby, 1980, 1981).

In the first part of this dissertation, reduction of external [Na^+] was shown to have a selective effect on increased release early in the time course of short conditioning trains at the frog neuromuscular junction. In this chapter, a slightly modified version of the model of stimulation-induced increases in transmitter release developed by Magleby and Zengel (1982) will be used to quantitatively describe sodium-induced changes in release in terms of the different components of increased release. The process of increased release described previously, which is selectively affected by manipulations of extracellular and intracellular Na^+ concentrations, will be shown to correspond in its kinetic properties to the first component of facilitation.

Materials and Methods

The preparation, solutions, stimulation paradigms, and recording techniques were the same as those described in Chapter 2 of this dissertation.

Modeling of V(t) data was done using a simplified form of the quantitative model for stimulation-induced increases in transmitter release described by Magleby and Zengel (1982). In this model, the effect of repetitive stimulation on the four components of increased release are assumed to result from changes in underlying factors in the nerve terminal which increase transmitter release. An impulse invading the nerve terminal is assumed to add an

increment to each of the underlying factors, which then decay with time.

Several models of increased release during short trains have been proposed (e.g., Mallart and Martin, 1967; Barrett and Stevens, 1972; Balnave and Gage, 1977; Zengel and Magleby, 1982). In general, facilitation can best be described by a power model of order 3 or 4 (Barrett and Stevens, 1972; Younkin, 1974; Zengel and Magleby, 1982). Power models of facilitation bear a close resemblance to the third- or fourth-power relationships between calcium and transmitter release that have been described at a variety of synapses by a number of workers (e.g., Dodge and Rahamimoff, 1967; Katz and Miledi, 1970). In this dissertation, a third-power relationship between the factors responsible for the first and second components of facilitation, or F_1 and F_2, and the observed increase in EPP amplitude due to facilitation, or F, was assumed for purposes of data analysis, giving the equation

$$F + 1 = (F_1^* + F_2^* + 1)^3 \qquad \text{(eqn. 3-1)}$$

where F_1^* and F_2^* represent the factors responsible for F_1 and F_2, the first and second components of facilitation.

The relationship between facilitation and augmentation was assumed to be multiplicative, as Zengel and Magleby (1982) have shown quantitatively at the frog neuromuscular junction. Potentiation has also been described by a multi-

plicative relationship to facilitation (Landau et al., 1973; Magleby, 1973). These relationships can be described by the equation

$$V + 1 = [F_1^* + F_2^* + 1]^3 [A + 1] [P + 1] \quad \text{(eqn. 3-2)}$$

where A is the magnitude of augmentation, and P is the magnitude of potentiation. The relationships described by this equation can successfully account for stimulation-induced changes in release under a wide variety of stimulus frequencies and conditioning train lengths (Magleby and Zengel, 1982).

Since the small magnitude and long time course of potentiation (tens of seconds to minutes) would allow no distinction from augmentation during the 10 impulse trains used in this study, these two processes of increased release were combined into a single term for modeling purposes. This assumption results in the simplified equation

$$V + 1 = [F_1^* + F_2^* + 1]^3 [A' + 1] \quad \text{(eqn. 3-3)}$$

in which A′ represents the magnitude of the combined augmentation and potentiation terms (hereafter referred to as augmentation).

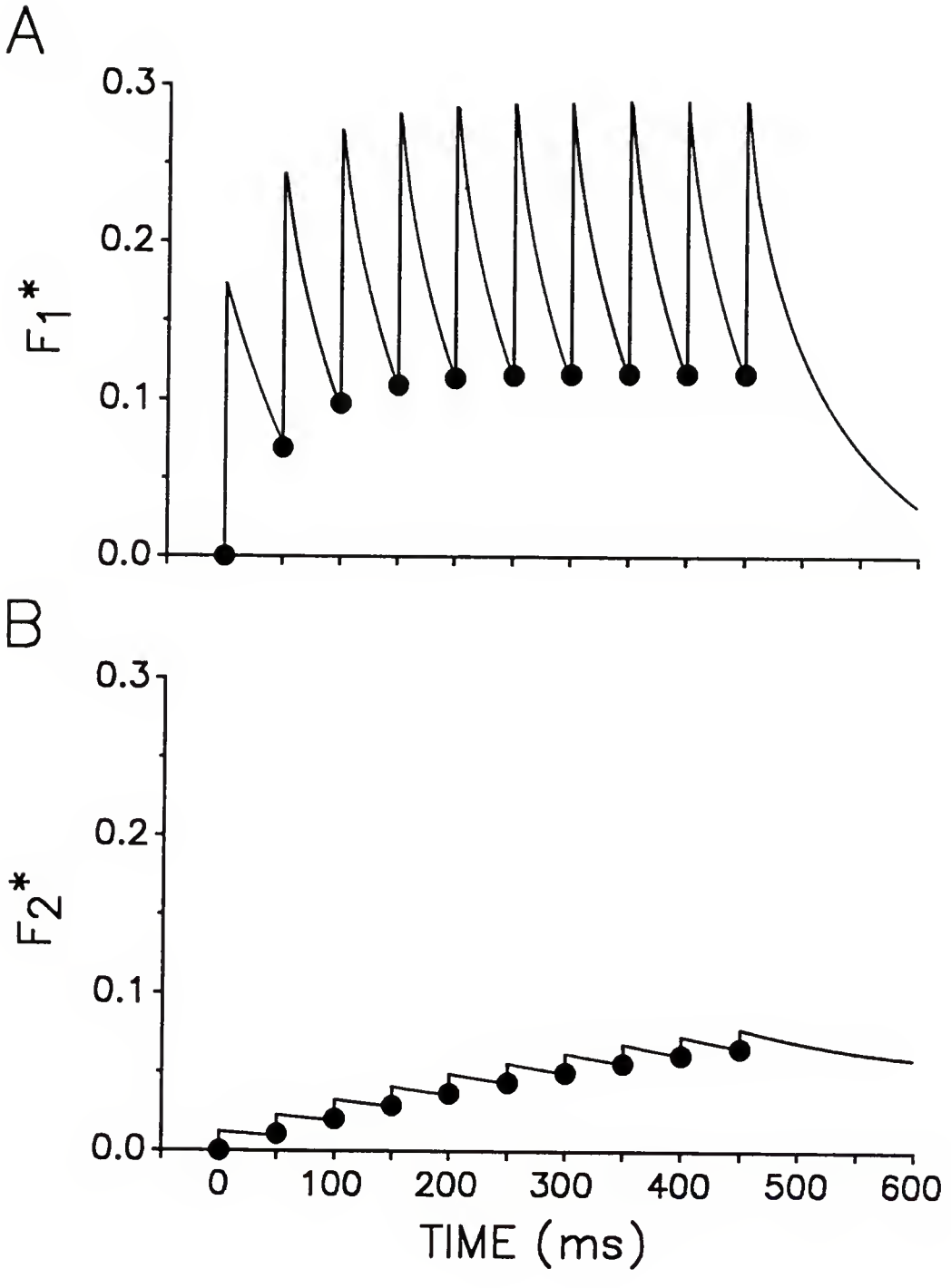
Increments in the factors underlying the two components of facilitation, which are added at the time of each impulse, were assumed to be constant during the train (Mal-

lart and Martin, 1967; Barrett and Stevens, 1972; Zengel and Magleby, 1982). The increment in augmentation added by each impulse has been found to increase with successive impulses during long conditioning trains of over 100 impulses (Zengel and Magleby, 1982). For analysis of 10 impulse trains, however, the increment in augmentation was assumed to remain constant as no significant difference in the magnitude of augmentation would have been apparent during such a short conditioning train.

Following a nerve impulse, the magnitudes of the factors underlying the two components of facilitation and augmentation decay with first-order kinetics with time constants of about 55 ms, 475 ms, and 7 s, respectively (Zengel and Magleby, 1982). Each component builds up during repetitive stimulation because there is insufficient time for the increment of each underlying factor added during a nerve impulse to decay before the next impulse invades the nerve terminal.

The basic assumptions of the model described above are summarized in Figure 3-1. In this diagram of the increase in total facilitation during a conditioning train, or F, the underlying factors F_1^* and F_2^* are each assumed to acquire a constant increment in magnitude (f_1^* and f_2^*, respectively) with each nerve impulse. Between impulses, the total magnitude of each component decays exponentially with a time constant which is approximately 55 ms for F_1^* and 475 ms for F_2^*. As can be seen in Figure 3-1A, the

FIGURE 3-1. Components of the model of stimulation-induced increases in transmitter release. Each component of increased release is assumed to be related to accumulation of some underlying factor in the nerve terminal, which is increased by a constant amount during a nerve impulse and which decays with first-order kinetics between nerve impulses. (A) Accumulation of F_1^*, the factor assumed to underlie the first component of facilitation. With each nerve impulse, a constant increment (f_1^*) is added to F_1^*. Model parameters are: f_1^*, 0.173; time constant of decay of F_1, 55 ms. (B) Accumulation of F_2^*, the factor assumed to underlie the second component of facilitation. As in (A) above, a constant increment (f_2^*) is added to F_2 with each nerve impulse. Model parameters are: f_2^*, 0.0119; time constant of decay of F_2, 475 ms. (C) Facilitation during 10 impulse trains, predicted by the power facilitation relation of equation 3-1, in which total facilitation, F, is related to the third power of F_1^* and F_2^*. (D) Modeling of increased release during 10 impulse conditioning trains. The filled circles depict data from the representative experiment shown in Figure 2-2A, which were measured during exposure of the preparation to a bathing solution containing 0.6 mM Ca^{2+} and 100% of normal [Na^+]. The best fit of the model (equation 3-3) to the data is shown by the solid line. Model parameters are: f_1^*, f_2^* as in (A) and (B); increment in augmentation (a'), 0.0118; time constant of decay of augmentation (A'), 7.0 s.


A
0.3
0.2
0.1
0.0
F1*
B
0.3
0.2
0.1
0.0
F2*
0
100
200
300
400
500
600
TIME (ms)

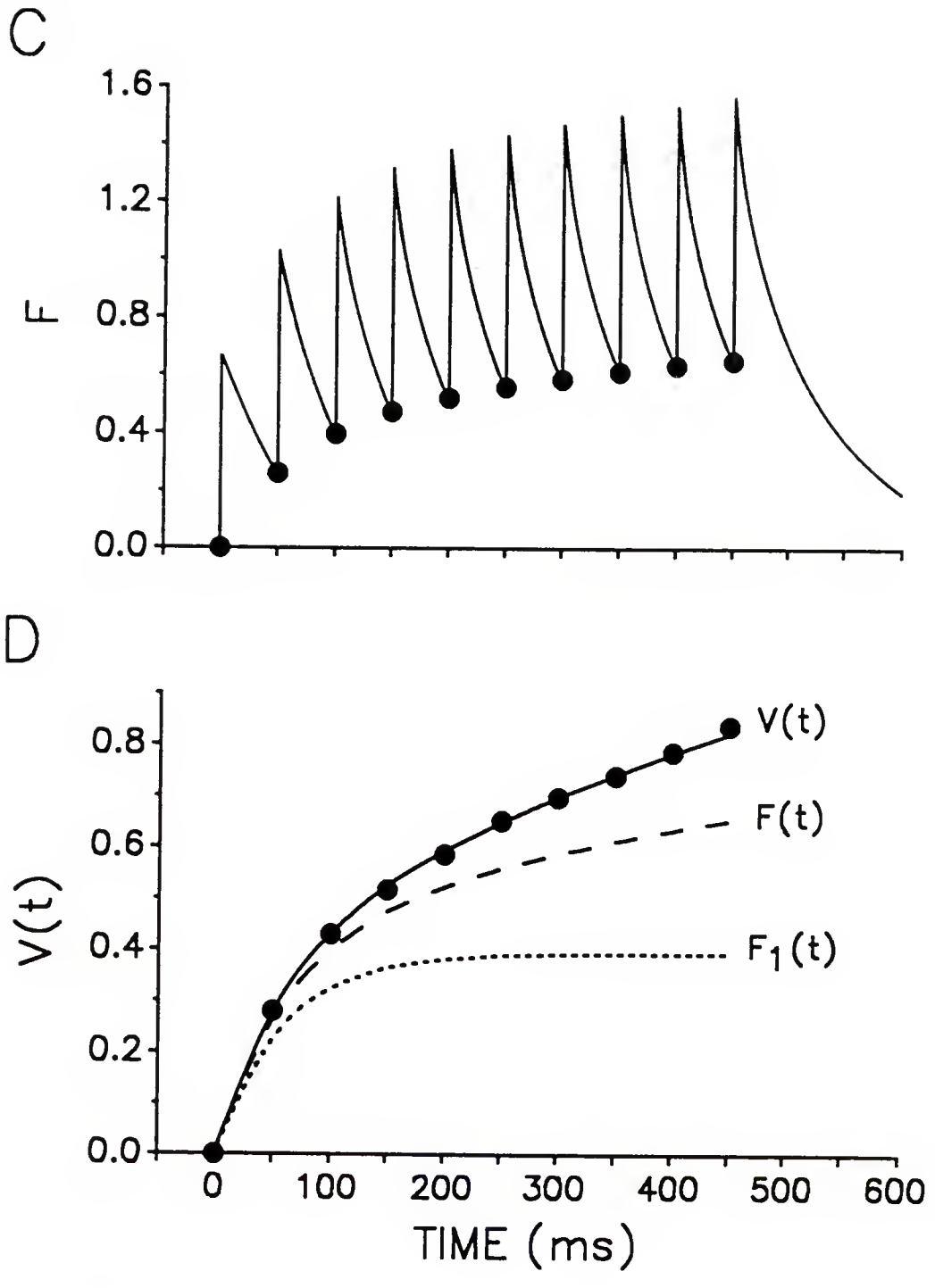


FIGURE 3-1 continued

magnitude of the increment f_1^* (represented by the vertical deflection at the time of each impulse) remains constant during the train. The magnitude of F_1^* present at the time of each stimulus has reached a plateau before the end of the 10 impulse train. Likewise, the magnitude of f_2^*, represented by the vertical deflection at the time of each impulse in Figure 3-1B, remains constant. However, because of the slower decay of F_2^*, the magnitude of F_2^* continues to increase throughout the train. The result of combining the effects of these two factors in a power facilitation model (see equation 3-1) is seen in Figure 3-1C. The increase of A′, or the term describing the combined effect of augmentation and potentiation, is not depicted but obeys similar considerations.

Data on V(t) obtained from the experiments presented in Chapter 2 were modeled using Lotus 1-2-3 graphic templates on an IBM PC computer. Unless otherwise noted, time constants of decay of the components of increased release were fixed at 55 ms for F_1, 475 ms for F_2, and 7.0 s for A′ (Zengel and Magleby, 1982), while the magnitudes of the increments f_1^*, f_2^*, and a′, the increment in the factor underlying the effects of augmentation and potentiation, were varied. As will be shown in this chapter, changes in the time constants of decay of the components of increased release could not adequately describe changes in V(t) due to experimental manipulations. The magnitudes of f_1^*, f_2^*, and a′ were chosen to minimize the sum of squared error

(SSE) of the difference between the model predictions and the actual V(t) data. The values for f_1^*, f_2^*, and a′ determined by this method were similar to published values for these parameters (Zengel and Magleby, 1982).

In some experiments, there was considerable variability in the data, such that a close fit giving reliable estimates of the model parameters was impossible to achieve. Data from an experiment were discarded from analysis if the minimum attainable SSE/V(450 ms) ratio exceeded 0.01. In order to determine whether this criterion for exclusion introduced a selection bias, modeling was also done on the averaged data from all experiments performed under a given experimental condition and the results compared with data from experiments analyzed separately. Sets of model parameters derived from these two methods of analysis differed by less than 5% in all cases, indicating that a selection bias was unlikely.

An example of data analyzed using the simplified model (equation 3-3) is shown in Figure 3-1D. The filled circles plot V(t) data from the representative experiment shown in Figure 2-2A, which were recorded from a preparation in a bathing solution containing 0.6 mM Ca^{2+} and 100% of normal $[Na^+]$. The solid line shows the predicted rise of V(t) obtained by using values of f_1^*, f_2^*, and a′ selected to give the best fit of the model predictions to the data ($f_1^* = 0.173$, $f_2^* = 0.0119$, $a' = 0.0118$). The relative contributions of the two components of facilitation and of

augmentation to V(t) are shown by setting f_2^* and a′ to zero (long-dashed line) and by setting only a′ to zero (short-dashed line). It is obvious that at 50 ms, the first component of facilitation makes the predominant contribution to V(t), while at later times during the train, the effects of the longer term components of increased release become more prominent.

Differences between the model parameters derived from best fits to experimental data were tested for statistical significance using Student's t tests.

Results

Effect of Reduced Extracellular Sodium on Components of Increased Release

In the previous chapter, reduction of extracellular $[Na^+]$ was shown to cause an increase in V(t) which was most prominent near the beginning of a 10 impulse conditioning train and appeared to approach a plateau by the end of the train. As the time course of this increase in V(t) is similar to that of the F_1 component of facilitation (Mallart and Martin, 1967; Zengel and Magleby, 1982), the V(t) data from the low-Na^+ experiments were modeled in order to test the hypothesis that the increase in V(t) seen with reductions in external $[Na^+]$ can be explained by a selective increase in the magnitude of the F_1 component of facilitation.

Figure 3-2 plots V(t) data collected in 100% Na^+ (filled circles) and 33% Na^+ solutions (open triangles)

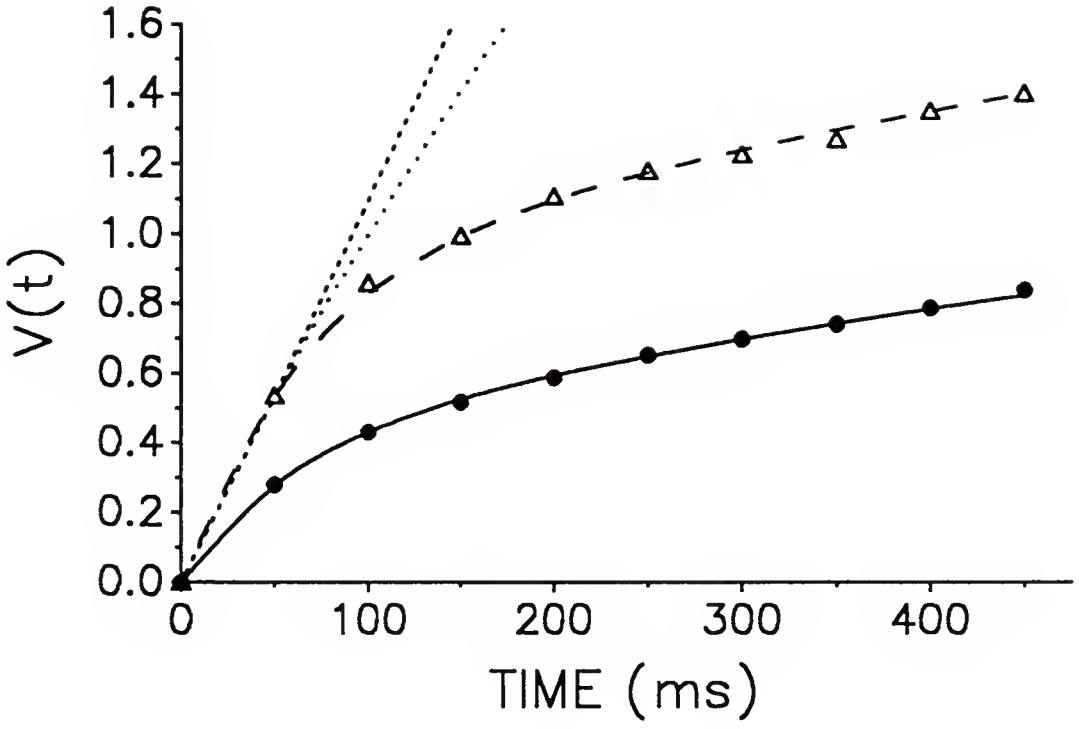


FIGURE 3-2. Modeling of the effect of reduced external $[Na^+]$ on stimulation-induced changes in release. V(t) data are from a single representative experiment (also shown in Figure 2-2) in which V(t) was measured under conditions of 100% (filled circles) and 33% (open triangles) of normal external $[Na^+]$. The bath contained 0.6 mM Ca^{2+}. The solid line represents the best fit of the model (described in the Methods) to the 100% Na^+ data, with the following parameters: $f_1^* = 0.173$, $f_2^* = 0.0119$, $a' = 0.0118$. The long-dashed line represents the best fit to the 33% Na^+ data, with $f_1^* = 0.341$, $f_2^* = 0.0119$, and $a' = 0.0118$. Selectively increasing the magnitude of f_2^* from 0.0119 to 0.0885 to account for the increase in V(50 ms) due to 33% Na^+ media resulted in a marked overprediction of all subsequent data points (dotted line). Assuming a selective increase in the time constant of decay of F_1 (from 55 ms to 225 ms) sufficient to account for the rise in V(50 ms) seen in the 33% Na^+ solution resulted in an even greater overprediction of subsequent data points (short-dashed line).

from the representative experiment shown in Figure 2-2A, along with model predictions fitted to the V(t) data. The 100% Na^+ data can best be described by the following parameters: $f_1^* = 0.173$, $f_2^* = 0.0119$, $a' = 0.0118$ (solid line). The model parameters are then changed to give the best fit to the V(t) data measured in a bathing medium containing 33% of normal NaCl. The low-$[Na^+]$ data can best be described by the following parameters: $f_1^* = 0.341$, $f_2^* = 0.0119$, $a' = 0.0118$ (long-dashed line). The change in V(t) attributable to reduced $[Na^+]_o$ could be described by changing only the magnitude of f_1^*, which was increased by 97%. It was typical of most preparations that the effect of reduced external $[Na^+]$ could be described quite well by a change in the magnitude of f_1^*, with only minor changes in the magnitudes of f_2^* and a' to improve the fit to the last few impulses of the train (these findings will be discussed in detail later in this chapter).

Changes in f_2^* or a' could not account for the data. For example, if a change in F_2 is assumed, and values for f_2^* chosen to fit the increase in V(50 ms) attributable to reduced external $[Na^+]$, the model drastically overpredicts V(t) by the end of the 10 impulse train, as is shown by the dotted line in Figure 3-2. Changes in a' chosen to account for the observed increase in V(50 ms) also lead to massive overpredictions by the end of the stimulus train (data not shown). A selective change in the time constant of decay of F_1 was also unable to account for the observed increase

in V(t), as is shown by the short-dashed line of Figure 3-2. A change in the time constant of decay of F_1 from 55 ms to 225 ms, chosen to account for the increase in V(50 ms) due to reduced external $[Na^+]$, consistently overpredicts V(t) for all subsequent impulses during the train.

As in the previous chapter, the effect of reduced $[Na^+]$ on V(t) appeared to be ion-specific. Modeling of V(t) data obtained in reduced $[Na^+]$ solutions in which N-methylglucamine chloride was used to maintain osmolality showed a similar result to modeling of V(t) data obtained when sucrose was used to maintain osmolality. The results are presented in Table 3-1, which presents estimates of the magnitudes of f_1^*, f_2^*, and a′ derived from 8 experimental preparations in bathing media containing 0.5 mM Ca^{2+}. These data show an increase of 38% in the magnitude of f_1^* (from 0.147 to 0.203) following reduction of extracellular $[Na^+]$ to 33% of normal with isosmotic substitution by sucrose. An increase (49%) was also noted upon reduction of $[Na^+]_o$ to 33% of normal with isosmotic substitution by N-methylglucamine. Small but statistically insignificant increases were also noted in the estimated magnitudes of f_2^* for the N-methylglucamine data, the importance of which is unclear at the present time.

It can be concluded that increases in V(t) due to reduced external $[Na^+]$ can most easily be explained by assuming a selective increase in the magnitude of the F_1 component of facilitation. No other single change in the

TABLE 3-1. EFFECT OF REDUCED EXTERNAL [Na^+] ON MODEL PARAMETERS DESCRIBING INCREASED RELEASE.

[Na^+] (% normal)	n	Substituted by[a]	Parameter Estimates (mean) f_1*	f_2*	a′
100	8	--	0.147	0.0086	0.0110
33	6	Sucrose	0.203[b]	0.0086	0.0110
33	8	NMG^+	0.220[c]	0.0126	0.0119

[a]External [Na^+] was reduced by removing NaCl and substituting with an isosmotic amount of either sucrose or N-methylglucamine chloride.
[b]Significantly different from 100% Na^+ data ($P < 0.005$).
[c]Significantly different from 100% Na^+ data ($P < 0.025$). Not significantly different from 33% NaCl/sucrose data ($P > 0.10$).

model parameters, either in the magnitudes of longer term components of increased release or in the time constant of decay of the F_1 component, appears to be adequate to describe the effect of low external $[Na^+]$ on V(t) during 10 impulse conditioning trains. A scenario in which more than one significant change occurs in the magnitudes or time constants of decay of the processes of increased release is not excluded by this analysis, but would be a less parsimonious explanation of the observed data.

Effect of Increased External Sodium on Components of Increased Release

Increasing the concentration of Na^+ in the bathing medium led to a small decrease in V(t), which could be described by a selective decrease in f_1^*, as is shown in Table 3-2. This table summarizes data from 6 preparations in bathing media containing 0.5 mM Ca^{2+}. The concentration of external Na^+ was alternated between 150% of normal and 100% of normal (with osmolality maintained by the addition of sucrose). The results were consistent with a selective reduction in the magnitude of f_1^* by an increase in external $[Na^+]$.

Comparison of the Effect of Reduced External Sodium with the Effect of Increased External Calcium on the Components of Increased Release

The effect of decreased $[Na^+]$ on V(t) appears to be qualitatively different from the effect of increased $[Ca^{2+}]$ on V(t), as has been reported previously (Mosier et al.,

TABLE 3-2. EFFECT OF INCREASED EXTERNAL [Na^+] ON MODEL PARAMETERS DESCRIBING INCREASED RELEASE.

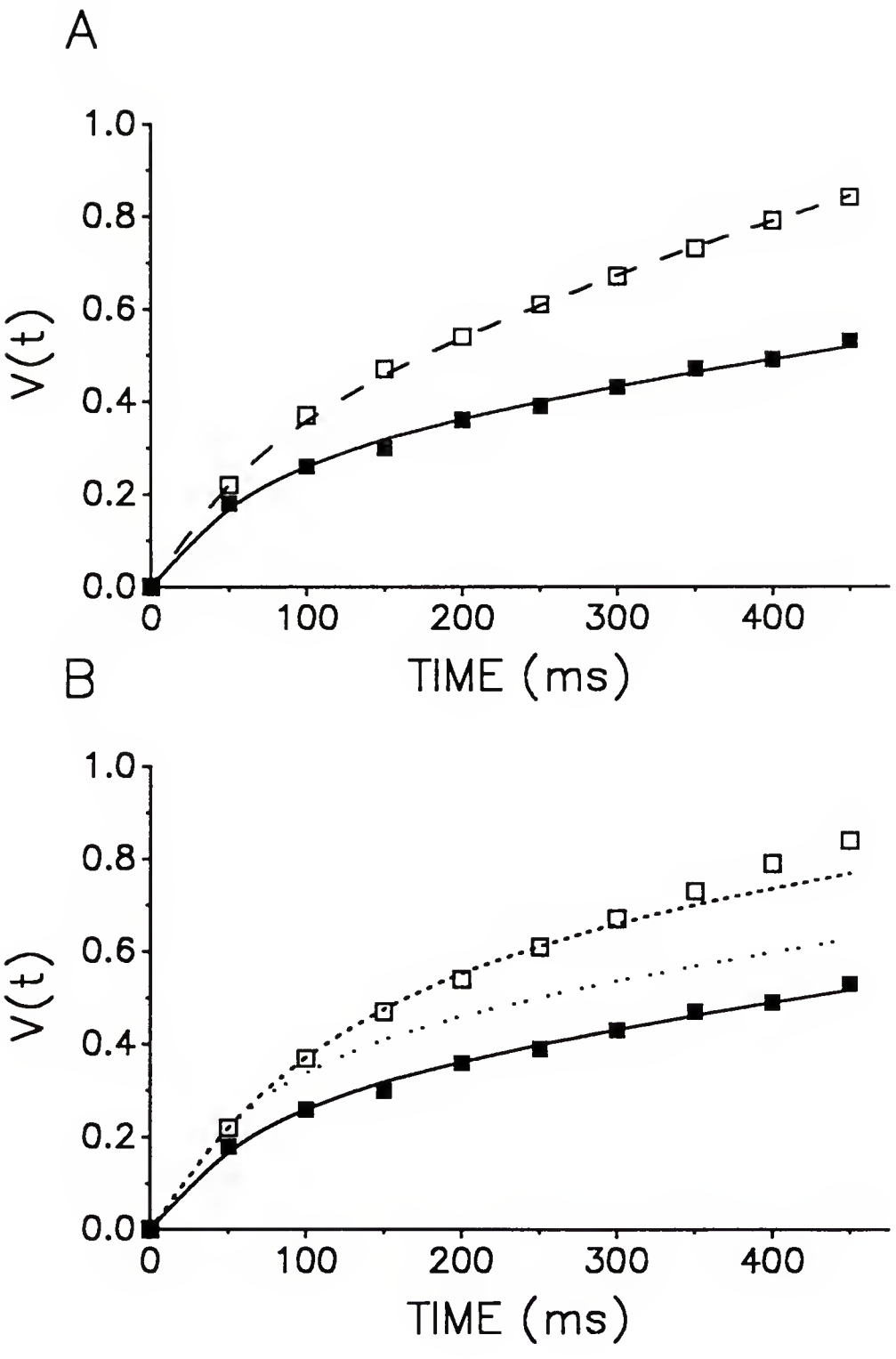
[Na^+] (percent normal)	n	Parameter Estimates (mean ± S.E.M.) f_1*	f_2*	a′
100[a]	6	0.124 ± 0.006	0.0054 ± 0.0007	0.0138 ± 0.0001
150	6	0.093 ± 0.006[b]	0.0063 ± 0.0009	0.0138 ± 0.0001

[a]Osmolality increased to that of the 150% Na^+ solution by the addition of sucrose.
[b]Significantly different from 100% Na^+ data ($P < 0.005$).

1986). In contrast to the sodium effect, the increase in V(t) caused by elevations in external [Ca^{2+}] could best be described by a selective increase in f_2*. To further illustrate this point, data from this laboratory showing V(t) during 10 impulse trains at two different external Ca^{2+} concentrations are shown in Figure 3-3A. This figure represents averaged data from 15 experiments conducted with 0.4 mM Ca^{2+} in the bathing solution (filled squares), and 10 experiments performed with 0.6 mM Ca^{2+} in the bathing solution (open squares). The best fit of the model (solid line) to the 0.4 mM Ca^{2+} data is obtained with the following parameters: f_1*, 0.103; f_2*, 0.0082; a′, 0.0110. The model parameters were then changed to give the best fit to the 0.6 mM Ca^{2+} data, resulting in the following values: f_1*, 0.114, f_2*, 0.0206; a′, 0.0106. The greatest change observed was in the magnitude of f_2*, which increased by 151%, while little or no change was observed in the magnitudes of the other incremental factors.

Changes in F_1 could not account for the Ca^{2+} effect. If the magnitude of f_1* was selectively increased to account for the increase in V(50 ms) seen in 0.6 mM Ca^{2+} solutions, all subsequent values of V(t) during the train were underpredicted (dotted line in Figure 3-3B). Alternatively, if a selective increase in the time constant of decay of the F_1 component were assumed to occur, a better fit could be obtained to V(t) for the first 5 impulses (short-dashed line in Figure 3-3B), but subsequent values

FIGURE 3-3. Modeling of the effect of increased external $[Ca^{2+}]$ on V(t) during 10 impulse trains. Data represent averaged V(t) values from 15 experiments in media containing 0.4 mM Ca^{2+} (filled squares) and 10 experiments in media containing 0.6 mM Ca^{2+} (open squares), also shown in Figure 2-14. (A) Best fit of the model to the 0.4 mM Ca^{2+} data, with parameters $f_1^* = 0.103$, $f_2^* = 0.0082$, and $a' = 0.0110$ (solid line). Parameters were then changed to give the best fit to the 0.6 mM Ca^{2+} data, with $f_1^* = 0.114$, $f_2^* = 0.0206$, $a' = 0.0106$ (long-dashed line). (B) Attempts to account for the Ca^{2+}-induced increase in V(t) by selective changes in other model parameters. The solid line represents the best fit of the model to the 0.4 mM Ca^{2+} data as in (A). Selectively increasing the magnitude of f_1^* (from 0.103 to 0.142) to account for the Ca^{2+}-induced increase in V(50 ms) resulted in underprediction of all subsequent data points (dotted line). A selective increase in the time constant of decay of F_1 (from 55 to 85 ms) to account for the Ca^{2+}-induced increase in V(50 ms) gave a good fit to data points early in the train, but underpredicted the last four points (short-dashed line).


A
1.0
0.8
0.6
0.4
0.2
0.0
V(t)
0
100
200
300
400
TIME (ms)
B
1.0
0.8
0.6
0.4
0.2
0.0
V(t)
0
100
200
300
400
TIME (ms)

of V(t) were underpredicted. In contrast, increasing the magnitude of a′ could also account for the increase in V(t) during during 10 impulse trains, but changes in a′ did not result in as good a fit (see Materials and Methods) as did changes in f_2^* (data not shown). Thus it appears that the increase in V(t) due to increased extracellular $[Ca^{2+}]$ can be explained by an increase in the magnitude of the second component of facilitation or of a longer-lasting component of increased release, and that this effect is distinct from the increase in V(t) which occurs in reduced extracellular $[Na^+]$.

External Calcium Dependence of the Effect of Reduced External Sodium on Components of Increased Release

In Figure 2-7 of the previous chapter, an increase in extracellular $[Ca^{2+}]$ was shown to increase the magnitude of the effect of reduced external $[Na^+]$ on V(t) in a manner which appeared to differ from the direct effect of increased external $[Ca^{2+}]$ on V(t). To determine whether these apparently disparate effects on V(t) could be explained by a change in a single underlying factor, the data presented in Figures 2-7 were modeled using the simplified version of the Zengel and Magleby model. The results obtained from modeling, which are presented in Table 3-3, show that at any given concentration of external Ca^{2+}, the magnitude of the estimated f_1^* increment appears to increase in a concentration-dependent manner with decreasing external $[Na^+]$. In addition, the magnitude of

TABLE 3-3. EFFECT OF EXTERNAL $[Na^+]$ AND $[Ca^{2+}]$ ON MODEL PARAMETERS DESCRIBING INCREASED RELEASE.

$[Ca^{2+}]$ (mM)	$[Na^+]$ (percent normal)	n	Parameter Estimates (mean ± S.E.M.) f_1*	f_2*	a′
0.4	100	14	0.159 ± 0.025	0.0118 ± 0.0030	0.0109 ± 0.0006
	50	7	0.187 ± 0.023[a]	0.0152 ± 0.0059	0.0111 ± 0.0004
	33	6	0.216 ± 0.041[b]	0.0123 ± 0.0052	0.0097 ± 0.0021
0.5	100	26	0.162 ± 0.022	0.0128 ± 0.0008	0.0107 ± 0.0014
	50	6	0.195 ± 0.010[c]	0.0139 ± 0.0039	0.0110 ± 0.0005
	33	17	0.231 ± 0.039[d]	0.0132 ± 0.0047	0.0106 ± 0.0017
0.6	100	14	0.153 ± 0.017	0.0137 ± 0.0050	0.0108 ± 0.0013
	50	7	0.197 ± 0.020[e]	0.0112 ± 0.0041	0.0107 ± 0.0021
	33	9	0.262 ± 0.062[f]	0.0149 ± 0.0062	0.0102 ± 0.0008

[a]Significantly different from 100% Na^+ data ($P < 0.025$). [b]Significantly different from 100% Na^+ data ($P < 0.005$). Not significantly different from 50% Na^+ data ($P > 0.05$). [c]Significantly different from 100% Na^+ data ($P < 0.005$). [d]Significantly different from 50% Na^+ data ($P < 0.025$) and from 100% Na^+ data $P < 0.005$). [e]Significantly different from 100% Na^+ data ($P < 0.005$). [f]Significantly different from 50% Na^+ data and from 100% Na^+ data ($P < 0.005$).

the low-$[Na^+]_o$ effect on f_1^* appears greater at higher concentrations of external Ca^{2+}. This can be more clearly seen in Figure 3-4, in which the ordinate represents the increase in f_1^* at a given concentration of external Na^+ over the value of f_1^* measured at normal external Na^+ concentrations. Although there is no apparent difference in the magnitude of the effect of reduced external $[Na^+]$ on f_1^* as external $[Ca^{2+}]$ rises from 0.4 mM to 0.5 mM, a further increase in $[Ca^{2+}]_o$ to 0.6 mM produces a striking increase in the magnitude of the low-$[Na^+]$ effect on f_1^*. This figure bears a remarkable resemblance to Figure 2-8, which shows an increase in the Na^+ effect on V(50 ms) in 0.6 mM Ca^{2+} media.

There appear to be no consistent changes in the effect of low external $[Na^+]$ on the magnitudes of f_2^* or a' during increases in external $[Ca^{2+}]$. Thus, the simplest explanation for the effect of increased external $[Ca^{2+}]$ on the magnitude of the low-$[Na^+]$ effect on V(t) appears to be a selective Ca^{2+}-dependence of the effect of reduced external $[Na^+]$ on the magnitude of f_1^*, the increment in the proposed factor underlying the first component of facilitation.

Effect of Monensin on Components of Increased Release

Addition of 2 uM monensin, an ionophore selective for Na^+ (Pressman and Fahim, 1982) to a bathing solution containing 0.5 mM Ca^{2+} caused a marked increase in V(t) which appeared early during a 10 impulse conditioning train

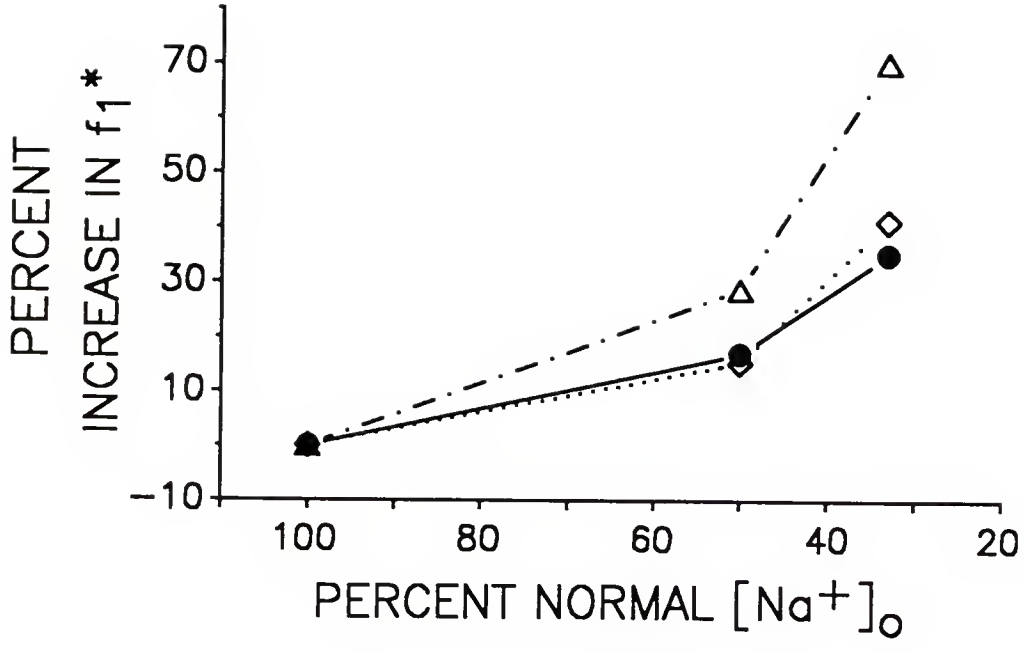


FIGURE 3-4. Effect of external $[Ca^{2+}]$ on the magnitude of the low-$[Na^+]$ effect on f_1*. Data represent plots of f_1* obtained from modeling the data from the experiments presented in Figure 2-7. Experimental data were collected in solutions containing 0.4 mM Ca^{2+} (filled circles), 0.5 mM Ca^{2+} (open diamonds), and 0.6 mM Ca^{2+} (open triangles). The ordinate of each data point represents the percentage increase in the value of f_1* over that obtained in 100% of normal $[Na^+]$ at the same concentration of bath Ca^{2+}. All f_1* values obtained from modeling the data collected in 100% Na^+ solutions have been normalized to zero. The non-normalized values of f_1* and tests of statistical significance are presented in Table 3-3.

and appeared to approach a plateau level by the end of the train. Figure 3-5 presents the V(t) data from a single preparation (also shown in Figure 2-12) bathed in solutions containing 0 and 2 uM monensin, along with the model predictions which gave the best fit to the observed V(t) values. In the control solution, the best fit was obtained with the following parameters: $f_1^* = 0.213$; $f_2^* = 0.0097$; $a' = 0.0111$. After the addition of monensin, the data could be best described by the following parameters: $f_1^* = 0.562$; $f_2^* = 0.0046$; $a' = 0.0111$. These parameter changes represent a 164% increase in the magnitude of f_1^* and a 53% decrease in the magnitude of f_2^*. No change in the magnitude of a' was necessary. The decrease in the magnitude of f_2^* was not characteristic of other preparations exposed to monensin. As with the increase in V(t) due to reduced extracellular $[Na^+]$, the increase in V(t) attributable to monensin could not be explained by an increase in the magnitude of f_2^* or in the time constant of decay of F_1 (data not shown). In 13 of 17 preparations in which 0.5-10 uM monensin was added to the bathing medium, the resulting change in V(t) could be explained by a selective increase in the magnitude of f_1^*. In the remaining 4 preparations, little or no increase in V(t) was observed. Thus the action of monensin on V(t) appears to be primarily on the increment of the factor underlying the first component of facilitation, an effect qualitatively similar to that seen with reduction of external $[Na^+]$.

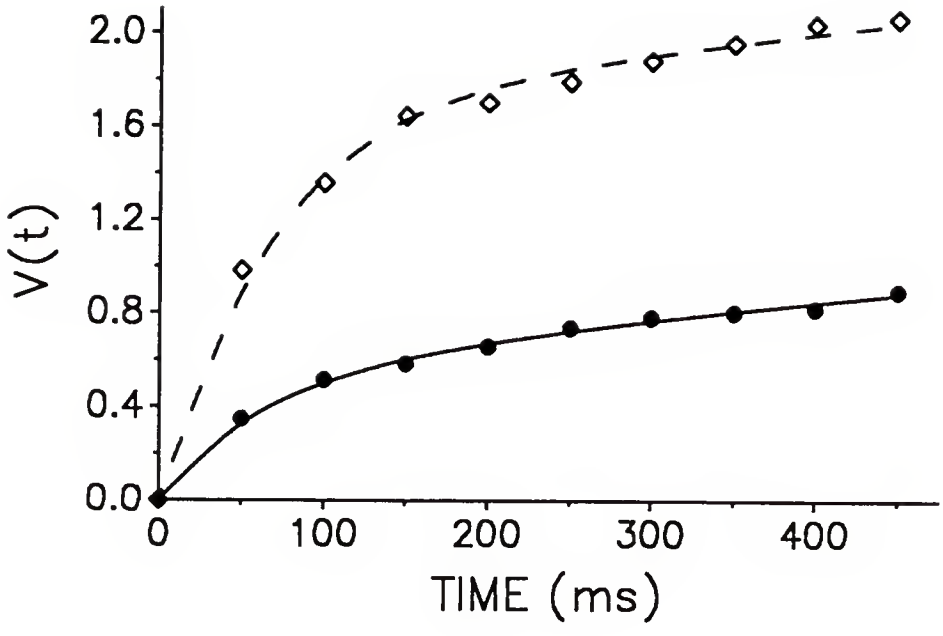


FIGURE 3-5. Modeling of the effect of the Na^+ ionophore monensin on V(t) during 10 impulse trains. Data are from a representative experiment performed at an external $[Ca^{2+}]$ of 0.5 mM (also shown in Figure 2-12). Data are from control solutions with no monensin (filled circles), and with 2 uM monensin added to the bathing medium (open diamonds). The solid line represents the best fit of the model to the control data, with the following parameters: $f_1^* = 0.213$, $f_2^* = 0.0097$, and $a' = 0.0111$. The model parameters were then changed to fit the V(t) data obtained in the solutions containing monensin (long-dashed line), with $f_1^* = 0.562$, $f_2^* = 0.0046$, and $a' = 0.0111$. A selective increase in the magnitude of f_2^* to account for the monensin-induced increase in V(50 ms) led to an overprediction of all subsequent data points (data not shown). The time constant of decay of F_1 could not be increased sufficiently to account for the change in V(50 ms) due to monensin.

Discussion

The major findings obtained from modeling the V(t) data presented in Chapter 2 are summarized as follows. For the model of stimulation-induced changes in release described in this chapter, reduction of $[Na^+]$ in the bathing solution led to an increase in the increment of the first component of facilitation during 10 impulse conditioning trains. The increase in f_1^* was concentration-dependent, becoming greater with larger reductions of external $[Na^+]$. The effect of reduced $[Na^+]$ on the magnitude of f_1^* appears to be specific for the sodium ion, as isosmotic substitution of reduced-$[Na^+]$ solutions with N-methylglucamine instead of sucrose led to a similar increase in the magnitude of f_1^*. Increasing external $[Na^+]$ has the opposite effect of reducing $[Na^+]_o$, apparently causing a selective decrease in the magnitude of f_1^*.

The effect of reduced external $[Na^+]$ on model parameters describing V(t) appears to be qualitatively different from the effect of increased external $[Ca^{2+}]$ on parameters describing V(t). While the effect on V(t) of changes in $[Na^+]_o$ can be described by a selective increase in the increment of the first component of facilitation, the effect on V(t) of changes in $[Ca^{2+}]_o$ can be best described by a selective change in the increment of the second component of facilitation. However, there appears to be a $[Ca^{2+}]_o$-dependence of the effect of reduced external $[Na^+]$ which is manifested by an increase in the effect of reduced

$[Na^+]$ on f_1^* in the presence of higher levels of external Ca^{2+}.

Monensin, a Na^+ ionophore which increases $[Na^+]_i$, selectively increases the magnitude of f_1^* when added to the bathing solution. This effect of monensin is similar to the effect of reduced external $[Na^+]$, which is known to result in a decrease in $[Na^+]_i$ (Thomas, 1972). As has been discussed in Chapter 2, the similar effects of reduced $[Na^+]_o$ and monensin on increased release suggest a mechanism which is dependent upon the transmembrane Na^+ gradient for its expression.

The effects of manipulations of Na^+ and Ca^{2+} concentrations on the parameters describing the processes of increased release in the modified model of Magleby and Zengel (1982) both qualitatively and quantitatively parallel the effects of the same manipulations on V(50 ms) which were described in Chapter 2. This suggests that the effects of changes in Na^+ concentrations on V(t) during short conditioning trains can be explained by the hypothesis that changes in Na^+ concentrations selectively affect the first component of facilitation.

Differential effects of changes in external $[Na^+]$ and external $[Ca^{2+}]$ on the components that act to increase transmitter release suggest that there are differences in the underlying mechanisms of action of these ions. These ions may modify release by acting differentially on various aspects of Ca^{2+} entry, sequestration, and extrusion from

the nerve terminal (reviewed in Blaustein, 1988), or on components of the release mechanism such as synaptic vesicles or release sites. Although not enough is known to suggest a specific mechanism for the second component of facilitation, the ionic dependence of the process of increased release described in the second chapter, together with modeling data suggesting an identity between this process and the first component of facilitation, support the hypothesis that the first component of facilitation may involve the action of a Na^+/Ca^{2+} exchange mechanism in the nerve terminal. This hypothesis is consistent with the assumptions of the residual-calcium hypothesis of increased release (Katz and Miledi, 1968; Zucker and Lara-Estrella, 1983). However, this hypothesis does not necessarily suggest that longer-lasting components of increased release exert their effect through residual Ca^{2+} remaining in the nerve terminal following an impulse. Indeed, the known properties and interactions of the four components of increased release at the frog neuromuscular junction are inconsistent with a simple fourth-power residual-calcium relationship underlying all four components of increased release (for discussion see Zengel and Magleby, 1982).

Conclusions

The results of modeling V(t), the fractional increase in transmitter release during conditioning trains of stimuli delivered to the frog sartorius nerve-muscle prepara-

tion, suggest that the selective increase in an early time course component of V(t) described in the first part of this dissertation can be explained by a selective effect of changes in external Na^+ concentration on the magnitude of the first component of facilitation. Consideration of the ionic selectivity and Na^+ gradient dependence of this process suggests that a Na^+/Ca^{2+} exchange mechanism may underlie the first component of facilitation. Model parameters describing the effect of external Ca^{2+} concentration on V(t) suggest that the increase in V(t) seen with increased external $[Ca^{2+}]$, which appears later during short conditioning trains than the increase in V(t) seen with reduced external $[Na^+]$, can best be explained by a selective increase in the magnitude of the second component of facilitation. These data provide further evidence that the two components of facilitation previously distinguished on the basis of their kinetic properties (Mallart and Martin, 1967; Zengel and Magleby, 1982) represent relatively independently-regulated processes which are distinguishable on the basis of their different ionic selectivities.

CHAPTER 4

EXPOSURE TO OUABAIN PROLONGS EVOKED TRANSMITTER RELEASE IN MEDIA CONTAINING VERY LOW CALCIUM CONCENTRATIONS

Introduction

Calcium ions have long been known to be essential in the process of neurotransmitter release. As the concentration of Ca^{2+} in the extracellular fluid is decreased, evoked acetylcholine release at the neuromuscular junction is diminished and eventually abolished (del Castillo and Stark, 1952; del Castillo and Katz, 1954a). The amount of transmitter released by a nerve impulse is extremely sensitive to extracellular Ca^{2+} concentrations, as has been shown by Dodge and Rahamimoff (1967), who demonstrated a fourth-power relationship between extracellular Ca^{2+} concentration and transmitter release. In other preparations, the amount of transmitter released during a nerve impulse has also been shown to vary as the third or fourth power of the extracellular Ca^{2+} concentration (Hubbard et al., 1968b; Katz and Miledi, 1970; Dudel, 1981). These effects of extracellular Ca^{2+} on evoked quantal transmitter release have been shown to occur regardless of the nature of the transmitter (e.g., Bracho and Orkand, 1970).

Calcium is not only essential for release, but it must be available during the period of depolarization produced

in the presynaptic terminal by an action potential. If calcium is applied to a nerve terminal after the period of depolarization, it has no effect on release (Katz and Miledi, 1967a).

The preceding experiments led to the calcium hypothesis of Katz and Miledi (1965, 1968), which proposed that extracellular Ca^{2+} actually enters the nerve terminal in order to evoke transmitter release. Subsequent experiments have strengthened the evidence for the calcium hypothesis. Depolarizing the nerve terminal to levels exceeding the reversal potential for Ca^{2+} inhibits transmitter release until termination of the depolarization, at which time release occurs after a short synaptic delay (Katz and Miledi, 1967b, 1977b; Llinas and Nicholson, 1975), which is consistent with the hypothesis that extracellular Ca^{2+} must enter the nerve terminal in order to cause evoked release. In addition, Llinas and Nicholson (1975), using the Ca^{2+}-sensitive photoprotein aequorin, demonstrated in presynaptic terminals of the squid giant synapse that evoked transmitter release was preceded by increases in the intracellular Ca^{2+} concentration. The magnitude of the presynaptic Ca^{2+} current measured at voltage-clamped squid nerve terminals has been shown to be linearly related to the extracellular Ca^{2+} concentration, whereas the postsynaptic response has been shown to vary according to the third or fourth power of the extracellular Ca^{2+} concentration (Augustine and Charlton, 1986). This result is con-

sistent with the hypothesis that the power relationship between extracellular [Ca^{2+}] and evoked transmitter release arises at a step (or steps) subsequent to Ca^{2+} entry.

Although the experiments of Katz and Miledi (1967b, 1977b) and Llinas and Nicholson (1975) suggest that extracellular Ca^{2+} entry is necessary to initiate transmitter release, they do not rule out a contribution of cytosolic Ca^{2+} to the release process. Cytosolic Ca^{2+} concentrations in the resting state are thought to be on the order of 100 nM, or four orders of magnitude less than the millimolar concentrations of Ca^{2+} found extracellularly (Dipolo et al., 1976). If cytosolic Ca^{2+} participates in the release process, a significant deviation from the level of release predicted by the fourth power of extracellular [Ca^{2+}] should be seen at very low quantal contents of release. This hypothesis was tested by Andreu and Barrett (1980), who found that a fourth-power relationship between extracellular [Ca^{2+}] and transmitter release existed even under conditions of very low quantal content. They suggested that the contribution of cytosolic Ca^{2+} to the release process was therefore likely to be insignificant.

Although the resting Ca^{2+} concentration within the cytosol may be very low (on the order of 100 nM), the total amount of intracellularly sequestered or stored calcium is considerable, approximately 99.9% of the total intracellular calcium measured by atomic absorption spectroscopy (Baker, 1978). Quantitative electron probe microanalysis

of rapidly frozen nerve terminals has shown total calcium concentrations of 0.4-1.2 mM in cytoplasmic organelles such as synaptic vesicles, mitochondria, and smooth endoplasmic reticulum (Andrews et al., 1987). If stored Ca^{2+} can be released from intracellular organelles by membrane depolarization, by accumulation of intracellular substances such as Na^{+}, or by pharmacological agents (reviewed in McBurney and Neering, 1987), a significant contribution of intracellular Ca^{2+} to release could occur.

A number of workers have suggested that under certain conditions, Ca^{2+} derived from intracellular sources may participate in the release process. Adam-Vizi and Ligeti (1984) have demonstrated increases in release of labeled acetylcholine from rat brain synaptosomes exposed to the calcium ionophore A23187 or to m-chloro-carbonylcyanid-phenylhydrazone (CCCP), an uncoupler of mitochondrial oxidative phosphorylation which causes Ca^{2+} efflux from mitochondria. These increases in acetylcholine release could be demonstrated even in the absence of membrane depolarization and external Ca^{2+}. Tetanic stimulation increases the frequency of miniature end-plate potentials (MEPPs) even when external $[Ca^{2+}]$ is reduced to levels less than 1 nM by the Ca^{2+} buffer EGTA (Miledi and Thies, 1971). Van der Kloot, Barton, and Cohen (1986), based on modeling of evoked release in frog neuromuscular junctions exposed to hypertonic bathing solutions (which elevate $[Ca^{2+}]_i$), have argued that elevated intracellular Ca^{2+} concentrations

may significantly affect measurements of the relationship between external [Ca^{2+}] and evoked release. These findings support the hypothesis that intracellular Ca^{2+} plays a significant role in the release process under certain conditions, especially those which increase intracellular [Ca^{2+}].

Application of the cardiac glycoside ouabain is one method of greatly increasing intracellular [Ca^{2+}], presumably by inhibition of the Na^+/K^+ pump and secondary inhibition of Na^+/Ca^{2+} exchange by collapse of the transmembrane Na^+ gradient (e.g., Deitmer and Schlue, 1983). In this study, the effect of ouabain on evoked release in the frog sartorius nerve-muscle preparation was investigated in solutions in which free Ca^{2+} was buffered to very low levels (<10 nM) with EGTA. The results described below demonstrate that in preparations exposed to ouabain, evoked release can occur in the absence of significant levels of extracellular Ca^{2+}.

Materials and Methods

The preparation, calcium-containing solutions, and recording techniques were the same as those described in Chapter 2 of this dissertation. No-added-calcium solutions were prepared with the following composition (in mM): NaCl 116, KCl 2, $MgCl_2$ 5, HEPES 2, glucose 5, and no added calcium. The free Ca^{2+} concentration of no-added-calcium solutions has been estimated to be on the order of 1 uM

(e.g., Hubbard et al., 1968a). As even very low concentrations of extracellular Ca^{2+} have been suggested to trigger Ca^{2+} release from intracellular stores in other systems (Fabiato, 1983; Lea and Ashley, 1989), experiments were performed under conditions in which the electrochemical gradient for Ca^{2+} entry into the cell was reversed by bathing preparations in media containing Ca^{2+} concentrations at least an order of magnitude lower than resting intracellular $[Ca^{2+}]$. To obtain bathing solutions capable of generating a reversed electrochemical gradient for Ca^{2+} (hereafter referred to as "calcium-free" solutions), the calcium buffer EGTA (1 mM) was dissolved in a no-added-calcium bathing solution prepared as above. This solution should have an external Ca^{2+} concentration of <10 nM (Portzehl et al., 1964; Hubbard et al., 1968a). The pH was adjusted to 7.2-7.4 with NaOH. The cardiac glycosides ouabain and strophanthidin, as well as the Ca^{2+} buffer EGTA, were obtained from the Sigma Chemical Company.

Two stimulation paradigms were employed: stimulation with conditioning trains of 10 impulses at a frequency of 20 impulses/s, delivered to the sartorius muscle nerve every 50 s, and stimulation with single impulses applied to the muscle nerve every 15 s. Both extracellular and intracellular recording techniques were used to detect end-plate responses (as described in Chapter 2).

The following general experimental approach was used in all experiments. One of the two stimulation paradigms

described in the preceding paragraph was selected for use throughout the entire experiment. End-plate potential amplitudes were measured in a control bathing solution (described in Chapter 2) using the selected stimulation paradigm. Following this determination, the bathing solution was replaced with either a no-added-calcium solution or a calcium-free solution to reduce evoked release to undetectable levels, and the time course of the elimination of evoked release was observed. When evoked end-plate potentials displayed on the oscilloscope screen were no longer apparent to visual inspection, the preparation was returned to the control bathing solution. After recovery of end-plate potential (EPP) amplitude to control levels, a test concentration of ouabain (2.8-20 uM) was added to the bathing solution. After a 15-90 min delay, EPP amplitude began to increase (described in Birks and Cohen, 1968a). When EPP amplitude reached a level of three times the pre-ouabain control amplitude, the bathing solution was replaced with the no-added-calcium solution or the calcium-free solution used earlier during the experiment. The time course of reduction of EPP amplitude was measured following this solution change. At very low levels of release, when failures of release due to quantal variation were common, 4-8 trials were averaged using a Nicolet 1170 signal averaging computer to detect the presence of evoked release.

When evoked release was directly assessed using intracellular recording techniques, it was necessary to be able

to distinguish between evoked, unitary end-plate potentials (the smallest EPPs recorded at a neuromuscular synapse, assumed to represent single quanta; Martin, 1966) and spontaneously occurring miniature end-plate potentials (MEPPs). This distinction became especially important during exposure of the preparation to calcium-free or no-added-calcium solutions following prolonged exposure to ouabain, when MEPP frequency has increased dramatically (Elmqvist and Feldman, 1965b; Birks and Cohen, 1968a). In order to prevent confusion of MEPPs with uniquantal EPPs at this time, the latencies of EPP response were measured from the end of the shock artifact from nerve stimulation to the beginning of the end-plate potential. During exposure of the preparation to calcium-free or no-added-calcium solutions following pretreatment with ouabain, when the uniquantal EPPs are common, only end-plate responses beginning within the previously determined range of response latencies (usually a time interval of 0.1-0.2 ms) were considered to represent evoked release. Using this criterion for determining evoked release, fewer than 10% of the responses thought to represent evoked release could be accounted for by spontaneous release events occurring within the measurement interval.

Analysis of extracellularly and intracellularly recorded data was performed as described in the Materials and Methods of Chapter 2.

Results

Ouabain, a cardiac glycoside which potently inhibits the Na^+/K^+ pump of the cell membrane, is known to also cause a profound increase in the concentration of cytosolic Ca^{2+} (e.g., Deitmer and Schlue, 1983). If intracellular Ca^{2+} is capable of participation in the release process, but its action is normally not detected due to its very low resting levels, application of an agent which increases intracellular Ca^{2+} may amplify the participation of intracellular Ca^{2+} in release to detectable levels. One way in which the participation of increased intracellular Ca^{2+} in release may be manifested is in the continuation of evoked release upon removal of external Ca^{2+} from the bathing medium.

In 8 experiments, the effect of ouabain on control EPP amplitude (the amplitude of the first end-plate potential in a train) was measured during 10 impulse trains delivered to the muscle nerve at an external $[Ca^{2+}]$ of 0.4 mM. After EPP amplitude was determined in the control solution, ouabain in a concentration of 2.8-20 uM was added to the preparation. Although prolonged exposure to ouabain had little or no effect on stimulation-induced increases in EPP amplitude in these experiments (described in Chapter 2), the control EPP amplitude began to increase dramatically after a delay of 15-80 min, consistent with the findings of Birks and Cohen (1968a). Continued exposure to ouabain resulted

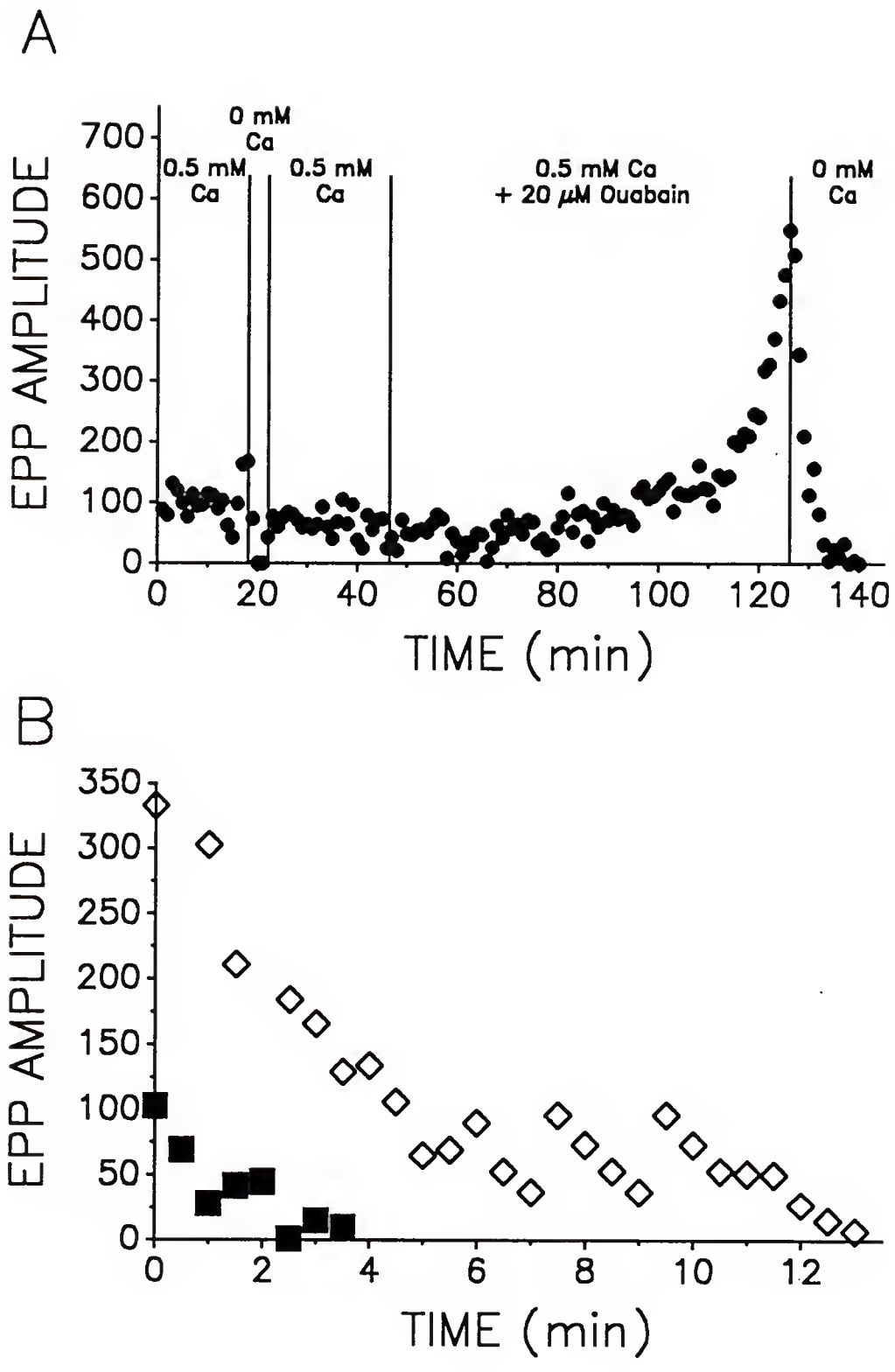
in EPPs of sufficient amplitude to evoke a muscle action potential, and/or failure of neuromuscular transmission. The latter effect was interpreted as blockade of axonal action potential conduction (Birks and Cohen, 1968a,b). Changing the bathing solution to a solution containing no added calcium dramatically reduced release in all preparations. In 4 of 8 preparations, however, release following exposure to ouabain was not abolished completely, but continued at a very low level for an extended period of time (15-54 min) before subsiding to undetectable levels. This surprising finding could not be explained by the time required for the preparation to equilibrate with the new solution (usually 2-3 min).

A number of experiments using single impulse stimulation patterns to measure control EPP amplitudes were performed using the general paradigm described in the Materials and Methods. As with the previous experiments, pretreatment with ouabain resulted in a prolonged period of evoked release in about one-third to one-half of preparations upon exposure to solutions containing no added calcium. Prolonged release could not be explained by the time required for equilibration of nerve-muscle preparations with the no-added-calcium bathing media, as exposure of preparations to no-added-calcium solutions prior to treatment with ouabain abolished detectable levels of release in every preparation within 4 min of completing the solution change. Substitution of calcium-free solutions (buffered

with 1 mM EGTA) for no-added-calcium solutions in the general experimental paradigm led to a similar prolongation of evoked release in 2 of 7 preparations. Although prolonged release was seen in a minority of preparations, the fact that it was observed at all suggests that the very small amounts of Ca^{2+} present in the no-added-calcium solutions do not explain the phenomenon of prolonged release.

Figure 4-1 depicts prolonged release in a representative extracellular experiment using no-added-calcium solutions. In Figure 4-1A, the mean EPP amplitude measured in the control solution has been normalized to 100, and all other EPP amplitude measurements scaled accordingly. As can readily be seen, control levels of EPP amplitude are stable before the 0.5 mM Ca^{2+} bathing solution is changed to a no-added-calcium solution. After the solution change, EPPs detectable by visual inspection of the oscilloscope screen are abolished within 2.5 min. Return of the preparation to the control Ringer's solution resulted in a slow return of EPP amplitude to control levels. Addition of 20 uM ouabain to the bathing solution caused no immediate change in EPP amplitude, but after about a 50 min delay, an accelerating increase in EPP amplitude began to be evident. When EPP amplitude reached a level of approximately 3 times the control level, the bathing solution was again changed to a no-added-calcium solution. Although EPP amplitude fell dramatically in the no-added-calcium bathing solution following exposure to ouabain, very small EPPs were detect-

FIGURE 4-1. Ouabain pretreatment causes prolonged release at very low external Ca^{2+} concentrations. Data are from a single experiment performed at an external [Ca^{2+}] of 0.5 mM in the control bathing solution. (A) Time course of EPP amplitude changes in bathing solutions containing 0.5 mM Ca^{2+}, no added Ca^{2+}, and 0.5 mM Ca^{2+} with 20 uM ouabain. The mean amplitude of EPPs recorded in the control bathing solution has been normalized to 100; other EPP amplitudes are scaled proportionally. (B) Time course of continued release during exposure to no-added-calcium bathing media before (filled squares) and after (open diamonds) an 80 min pretreatment with 20 uM ouabain. The pre-ouabain EPP amplitude measured just prior to removal of Ca^{2+} has been normalized to 100; other EPP amplitudes are scaled proportionally.


A
EPP AMPLITUDE
700
600
500
400
300
200
100
0
0 mM Ca
0.5 mM Ca
0.5 mM Ca
0.5 mM Ca + 20 μM Ouabain
0 mM Ca
0
20
40
60
80
100
120
140
TIME (min)
B
EPP AMPLITUDE
350
300
250
200
150
100
50
0
0
2
4
6
8
10
12
TIME (min)

able until 12 min after the solution change. The time course of release in no-added-calcium solutions can be better seen in Figure 4-1B, which plots EPP amplitudes (from the experiment in Figure 4-1A) over time following the introduction of a no-added-calcium solution. In this figure, EPP amplitudes prior to ouabain treatment (filled squares) are normalized to 100, and EPP amplitudes after ouabain treatment (open diamonds) are scaled accordingly. Although an initial decline in release occurs under both conditions, evoked release is clearly maintained at a low level for a prolonged period of time after pretreatment with ouabain.

Intracellular experiments were also performed to confirm the results of the extracellular studies. In three experiments, bathing the preparation in a no-added-calcium solution abolished release within 2-3 min. As can be seen in the representative experiment depicted in Figure 4-2, exposure to ouabain resulted in a parallel increase in both the quantal content of release (measured as described in the Materials and Methods section of Chapter 2) and the EPP amplitude, confirming that the increase in EPP amplitude induced by ouabain exposure results from an increase in transmitter release. At the same time, an accelerating increase in the frequency of MEPPs occurred. This elevation of spontaneous release (usually to a frequency of >200/s, from a resting frequency of about 1/s) continued even in the virtual absence of external calcium and/or

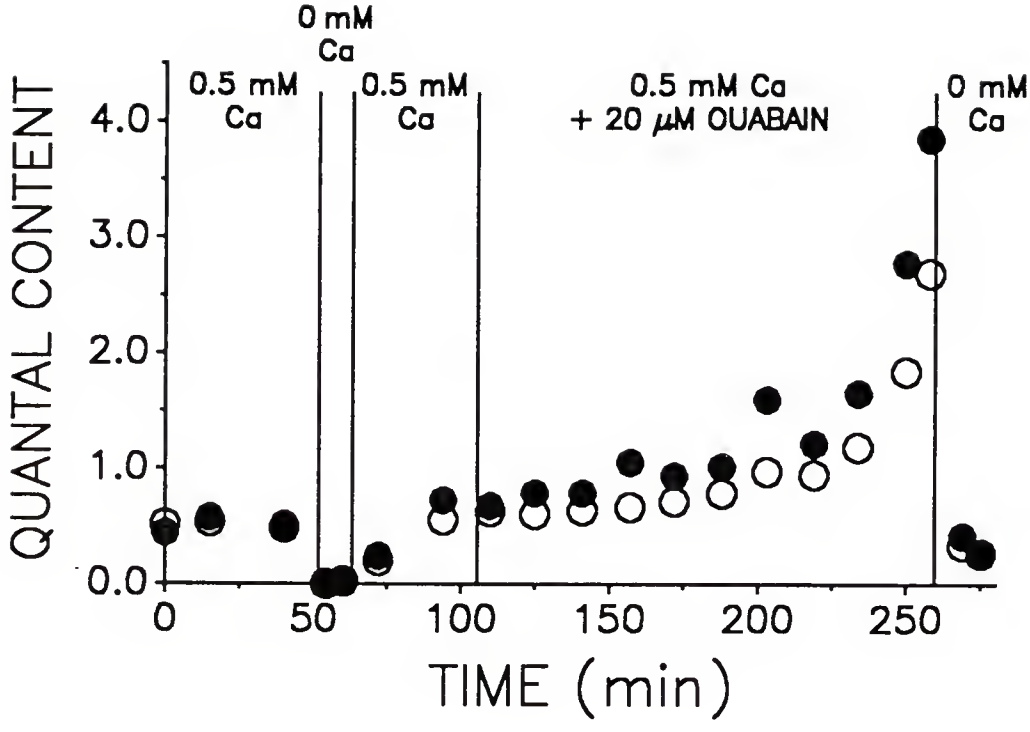


FIGURE 4-2. Intracellular recording of continued transmitter release at very low external [Ca^{2+}] following pretreatment with ouabain. Data are from a representative preparation started at an external Ca^{2+} concentration of 0.5 mM. Quantal contents were calculated as described in the Methods section of Chapter 2. Filled circles represent averages of quantal contents determined by the method of failures and the method of coefficient of variation. Open circles represent quantal contents derived from ratios of mean EPP amplitude to mean MEPP amplitude. As MEPP amplitude remained constant during the experiment, the latter measure of quantal content may also be interpreted as an index of EPP amplitude. During the interval from 56 min to 61 min, the bathing medium was replaced with a no-added-calcium solution. Evoked release was abolished within 2 min. At 105 min, 20 uM ouabain was added to the bathing medium. At 258 min, the bathing medium was again replaced with a no-added-calcium solution. Evoked release could be measured for 17 min following the solution change.

failure of axonal conduction into the nerve terminal (Birks and Cohen, 1968a; Baker and Crawford, 1975). Bathing the preparation in no-added-calcium Ringer's solution following ouabain exposure drastically reduced the amount of evoked release, but in 2 of 3 preparations, evoked release continued to be detectable for approximately 10 min following the solution change.

Discussion

The generally accepted view of the calcium hypothesis of release is that extracellular Ca^{2+} entry through Ca^{2+} channels virtually determines the amount of transmitter released during a nerve impulse. Intracellular Ca^{2+}, or other factors such as membrane voltage, have been assumed by most investigators to play little or no direct role in the release process. However, this study demonstrates that after pretreatment with ouabain, a cardiac glycoside known to increase intracellular $[Ca^{2+}]$, evoked release can occur at concentrations of extracellular calcium which are at least three orders of magnitude below the minimum concentrations necessary to support evoked release in untreated preparations. This finding directly contradicts the generally accepted view of the role of extracellular Ca^{2+} in release, while remaining consistent with the idea that the concentration of Ca^{2+} at or near the release site determines the level of transmitter release.

In very low $[Ca^{2+}]_o$ solutions, prolongation of evoked transmitter release occurred in only one-third of ouabain-treated preparations. Several factors may have contributed to the inconsistency in the appearance of prolonged evoked release. Ouabain has been shown by a number of investigators (e.g., Birks and Cohen, 1968a; Baker and Crawford, 1975) to cause failure of axonal conduction in frog neuromuscular preparations after prolonged exposure. In several of the experiments described above, failure of neuromuscular transmission occurred even before changing the bathing medium to a very low Ca^{2+} solution, consistent with the hypothesis that failure of axonal conduction may have contributed to an early decline in evoked release from some preparations. In three preparations, the nerve terminal action potential could be recorded extracellularly prior to ouabain treatment. Following the first change to a calcium-free solution and abolition of evoked release, the nerve terminal action potential was easily visible, indicating that neuromuscular transmission had failed at a step subsequent to action potential invasion of the nerve terminal. However, during the second exposure to a calcium-free solution (following ouabain pretreatment), evoked release was quickly abolished and the nerve terminal action potential could no longer be detected, indicating that axonal action potential conduction had been compromised. A second factor contributing to inconsistency in the appearance of prolonged evoked release may have been the very low exter-

nal Ca^{2+} concentrations used in the experimental paradigm, which have been noted to cause a marked destabilization of responses from neuromuscular preparations even in the presence of considerable amounts of external Mg^{2+} (Andreu and Barrett, 1980). A third factor contributing to inconsistency in the appearance of prolonged release may simply be variability between preparations, possibly due to differences in the calcium uptake and/or extrusion mechanisms of different nerve terminals (e.g., Grinnell and Herrera, 1981). Fortunately, the significance of prolonged evoked release following pretreatment with ouabain does not rest on the consistency of its occurrence, but on the fact that it occurs at all.

The work of many investigators has pointed toward the possibility of factors other than extracellular Ca^{2+} entry through Ca^{2+} channels as participants in the transmitter release process. Ouabain has been shown to induce release of labeled acetylcholine from synaptosome preparations in the virtual absence of external Ca^{2+} (Adam-Vizi and Ligeti, 1984; Blasi et al., 1988). Spontaneous release from motor nerve terminals is also increased by ouabain, probably via an indirect inhibition of Na^{+}/Ca^{2+} exchange (Molgo et al., 1987). Glusman and Kravitz (1982) reported that the neurohormone serotonin partially restored nerve-evoked transmitter release at the lobster opener muscle neuromuscular junction in low-Ca^{2+} media (10^{-8} M). To my knowledge, however, the experiments described in this dissertation

represent the first time that evoked transmitter release has been demonstrated in a vertebrate synapse in the virtual absence of external Ca^{2+}.

One major participant in the release process is likely to be intracellular Ca^{2+}. The underlying assumption of the residual calcium hypothesis of stimulation-induced increases in transmitter release (Katz and Miledi, 1968) is that Ca^{2+} which has not been completely sequestered or extruded following a nerve impulse may interact with Ca^{2+} entering during a subsequent nerve impulse to increase release. The contribution of intracellular Ca^{2+} to release is also unlikely to be due entirely to free cytosolic Ca^{2+}. By analogy with the Ca^{2+} release mechanism of the sarcoplasmic reticulum of skeletal muscle, the smooth endoplasmic reticulum of the nerve terminal has been proposed to release stored Ca^{2+} when activated by Ca^{2+} entry during a nerve impulse (e.g., McBurney and Neering, 1987; Blaustein, 1988).

Other processes may also contribute to the phenomenon of evoked release in the virtual absence of extracellular Ca^{2+}. Dudel et al. (1983) have suggested that depolarization of the nerve terminal by an action potential may itself trigger release, but this hypothesis has not been widely accepted (e.g., Zucker and Lando, 1986). Although a slow type of transmitter release which is voltage-dependent but calcium-independent has been described in teleost retinal horizontal cells (Schwartz, 1987), it is thought that

this type of release is too slow to account for evoked release at neuromuscular junctions. However, the findings presented in this study are not inconsistent with some other voltage-mediated mechanism of release.

Magnesium ions have also been implicated as participants in aspects of evoked release. Although extracellular Mg^{2+} acts as a competitive antagonist to Ca^{2+} entry, Mg^{2+} may participate in evoked release if it can gain access to the intracellular environment, either through Ca^{2+} channels under conditions of reduced extracellular Ca^{2+} or by introduction through Mg^{2+}-containing liposomes (Andreu and Barrett, 1980; Lev-Tov and Rahamimoff, 1980; Kharasch et al., 1981). Although the same concentration of Mg^{2+} is present in the bathing medium throughout the experiment, it is possible that ouabain may somehow affect sensitivity of the release process to Mg^{2+}, or influence Mg^{2+} uptake and extrusion mechanisms in the nerve terminal.

A fourth possible explanation for prolongation of evoked release is based on a concept advanced by Silinsky (1985), which states that evoked release is simply the synchronization of spontaneous release events. According to this hypothesis, the factors responsible for triggering release do not actually increase release, but merely act to synchronize it. Recent evidence for this hypothesis comes from the work of Molgo et al. (1989), who showed that application of botulinum toxin type D to the rat neuromuscular junction resulted in abolition of evoked end-plate

potentials and the replacement of EPPs by a period of high-frequency miniature end-plate potentials following each nerve action potential. Molgo et al. (1989) interpreted these results as evidence for a process acting to synchronize quantal release, which was distinguishable from the process of transmitter release itself. If this hypothesis proves to be accurate, it is possible that the evoked release seen in this study represents the synchronization of the greatly elevated number of spontaneous release events which has been observed upon exposure to ouabain (e.g., Baker and Crawford, 1975). This explanation for prolonged evoked release is not necessarily inconsistent with the previously discussed factors which may participate in the release process.

Ouabain itself may have effects other than those attributable to its effect on intracellular $[Ca^{2+}]$. It is possible that the increase in intracellular $[Na^{+}]$ produced by blockade of the Na^{+}/K^{+} pump (Thomas, 1972; Ellis and Deitmer, 1978; Deitmer and Schlue, 1983) may have a direct effect on transmitter release, as has been suggested by several investigators (Rahamimoff et al., 1980; Lev-Tov and Rahamimoff, 1980).

Conclusions

Exposure of frog sartorius nerve-muscle preparations to the cardiac glycoside ouabain results in a marked prolongation of evoked transmitter release in the virtual absence

of external Ca^{2+} in a number of preparations. This finding suggests that the generally accepted view of the calcium hypothesis of release, which assumes that the rise in intracellular Ca^{2+} believed to trigger release is derived exclusively from the extracellular medium, may have to be revised. Under conditions of very low external [Ca^{2+}], some other factor(s), possibly Ca^{2+} derived from intracellular sources, may be sufficient to mediate evoked release.

CHAPTER 5

CONCLUSIONS

Characterization of the properties of neurotransmitter release can increase our understanding of the release process itself. Two of these properties, the stimulation-induced increase in release and the calcium dependence of release, are described in this dissertation. Until these properties of the release process are accounted for in terms of specific underlying mechanisms, the release process will not be fully understood.

Residual Calcium and the Components of Increased Release

At least four kinetically distinct components of stimulation-increased release have been described at the frog neuromuscular junction (Magleby and Zengel, 1982). The data presented in this dissertation provide further evidence for the hypothesis that these four components of increased release represent the effects of underlying biochemical or biophysical mechanisms which act to increase transmitter release during repetitive stimulation. Two components of increased release, augmentation and the second component of facilitation, have been shown to be enhanced selectively by Ba^{2+} and Sr^{2+}, respectively (Zengel et al., 1980). The experiments described in this dissertation, together with previous results from this laboratory

(e.g., Mosier et al., 1986), suggest that two components of increased release, the first component of facilitation and a longer term component (perhaps the second component of facilitation), are selectively affected by changes in external Na^+ and Ca^{2+} concentrations, respectively.

According to the residual calcium hypothesis of Katz and Miledi (1968), increases in transmitter release result from accumulation of Ca^{2+} in the nerve terminal during repetitive stimulation. However, a simple model relating increases in residual intraterminal Ca^{2+} concentration to all four components of increased release fails to account for the differential effects of extended stimulation and time on the decay constants of the four components of increased release (Magleby and Zengel, 1982). Furthermore, the differential effects of Na^+ and the divalent cations Ca^{2+}, Sr^{2+}, and Ba^{2+} on components of increased release are inconsistent with a single mechanism underlying increases in release. The above difficulties with the residual calcium hypothesis as originally proposed by Katz and Miledi (1968) have led to the proposal that the different components of increased release result from the accumulation of Ca^{2+} or Ca^{2+}-activated factors in two or more different intraterminal "pools" which may act somewhat independently to increase transmitter release (e.g., Blaustein, 1988). Perhaps other ions such as Mg^{2+} also accumulate and act on the release mechanism (Kharasch et al., 1981).

The residual calcium hypothesis in its original formulation relates increased transmitter release to the amount of residual Ca^{2+} persisting in the nerve terminal after an action potential. It would predict that the amount of increased release would be correlated with the control level of release. The findings presented in this dissertation, that a substantial enhancement of increased release can occur without an increase in the control level of release, argue against this formulation of the residual calcium hypothesis. This represents additional evidence for the view that different mechanisms may underlie different aspects of increased release.

The Sodium-Calcium Exchange Mechanism and Processes of Increased Release

Calcium ions entering the nerve terminal during a depolarization cause an increase in the intracellular Ca^{2+} concentration which is believed to decay during repolarization with a time constant of less than 1 ms (Llinas et al., 1981). Buffering of Ca^{2+} by plasma proteins and diffusion of Ca^{2+} away from the release site are thought to underlie this rapid reduction of intracellular Ca^{2+}, as the rates of Ca^{2+} sequestration and extrusion from the nerve terminal are too slow to account for such a rapid decay of intraterminal Ca^{2+} concentrations (Rasgado-Flores and Blaustein, 1987b). Nevertheless, all Ca^{2+} that enters during an action potential must eventually be extruded from the nerve terminal if the cell is to remain in a steady state.

The concentration of intracellular Ca^{2+} is normally maintained at very low levels (on the order of 100 nM; Baker et al., 1971; Dipolo et al., 1976). Two mechanisms of Ca^{2+} extrusion across the plasma membrane have been defined, an ATP-driven Ca^{2+} pump and a Na^+/Ca^{2+} exchanger, the former of which is insensitive to large changes in extracellular Na^+ (reviewed in Dipolo and Beauge, 1983). The Na^+/Ca^{2+} exchanger uses energy from the transmembrane Na^+ electrochemical gradient to extrude Ca^{2+} from the cytosol. In several different preparations including guinea pig ventricular myocytes, barnacle muscle cells, and salamander rod outer segments, about 3 Na^+ ions are exchanged for each Ca^{2+} ion, making the exchange process electrogenic and imparting membrane potential sensitivity to the exchanger (Kimura et al., 1986; Rasgado-Flores and Blaustein, 1987a; Lagnado et al., 1988). The exchanger is capable of bidirectional Ca^{2+} transport; it can also transport Ca^{2+} into the cell when the Na^+ concentration gradient is decreased or when the membrane is depolarized (Dipolo and Beauge, 1987).

Early studies of the Na^+-dependent Ca^{2+} efflux mechanism in squid axons demonstrated a significant contribution of the Na^+/Ca^{2+} exchanger to the maintenance of resting intracellular Ca^{2+} concentrations (e.g., Baker et al., 1971). However, the Na^+/Ca^{2+} exchanger does not appear to contribute to the regulation of resting Ca^{2+} concentrations in abdominal ganglion neurons of Aplysia, indicating that

this mechanism is not essential to Ca^{2+} homeostasis in the resting cell (Levy and Tillotson, 1988).

The major plasmalemmal Ca^{2+}-transporting mechanisms, the ATP-driven Ca^{2+} pump and the Na^{+}/Ca^{2+} exchanger, are believed to operate in parallel to regulate intracellular Ca^{2+} concentrations (reviewed in Dipolo and Beauge, 1983). The ATP-dependent Ca^{2+} pump has a relatively high apparent affinity for intracellular Ca^{2+} (K_{Ca} = 200 nM), but a limited capacity for Ca^{2+} transport (about 200 fmol cm^{-2} sec^{-1}; Dipolo and Beauge, 1983). In contrast, the Na^{+}/Ca^{2+} exchange mechanism possesses a lower affinity for Ca^{2+} than the Ca^{2+} pump (K_{Ca} = 0.5-1.0 uM), ensuring that its influence on levels of intracellular Ca^{2+} is minimal in the resting state (Sanchez-Armass and Blaustein, 1987). However, in the presence of higher concentrations of intracellular Ca^{2+} such as those seen during repetitive stimulation (1 uM or greater; Llinas et al., 1981), the relatively high capacity of the Na^{+}/Ca^{2+} exchanger for Ca^{2+} transport (about ten times that of the ATP-driven Ca^{2+} pump) would favor a significant contribution of the exchanger to intracellular Ca^{2+} homeostasis (Dipolo and Beauge, 1983; Sanchez-Armass and Blaustein, 1987).

Further evidence favoring the view described above is the observation that the turnover rate of the exchanger is sensitive to the intracellular Ca^{2+} concentration, increasing dramatically as the intracellular Ca^{2+} concentration increases to the range of 1 uM (Dipolo and Beauge, 1986;

Rasgado-Flores and Blaustein, 1987b). Under resting conditions, even when the electrochemical gradient favors Ca^{2+} extrusion by the exchanger, the turnover rate of the exchange may be so low as to preclude a significant contribution of the exchange to Ca^{2+} regulation in the resting neuron (Blaustein, 1988). Therefore, the greatest contribution of the Na^{+}/Ca^{2+} exchanger to neuronal Ca^{2+} regulation may be during periods of repetitive stimulation, when the intraterminal Ca^{2+} concentration is increased and the ATP-dependent Ca^{2+} pump is saturated, rather than during the resting state.

In addition to Ca^{2+} extrusion, the Na^{+}/Ca^{2+} exchanger may contribute to the entry of Ca^{2+} into the cell. Because the exchange mechanism can operate in a bidirectional manner, a net Ca^{2+} influx may occur via the exchanger whenever the membrane potential exceeds the reversal potential for the Na^{+}/Ca^{2+} exchanger, as is seen during the action potential when the membrane is depolarized and the concentration of intracellular Ca^{2+} is elevated (Blaustein, 1988). Such an entry of Ca^{2+} via the Na^{+}/Ca^{2+} exchanger has recently been described during the action potential in guinea pig ventricular myocytes (Egan et al., 1989). It is conceivable that Ca^{2+} entering the nerve terminal via the exchanger, although small in comparison to the amount of Ca^{2+} entering through voltage-gated Ca^{2+} channels, may contribute to the release process or to stimulation-induced increases in transmitter release.

Due to the evidence supporting a role for the Na^+/Ca^{2+} exchanger in neuronal Ca^{2+} regulation during periods of increased neuronal activity, many investigators have proposed that the Na^+/Ca^{2+} exchanger may contribute to one or more of the components of increased release seen with repetitive stimulation (Parnas et al., 1982; Meiri et al., 1986; Misler et al., 1987). Most investigation has focused on the role of the exchanger in longer term components of increased release such as potentiation (Meiri et al., 1986; Misler et al., 1987; Nussinovitch and Rahamimoff, 1988). However, Sanchez-Armass and Blaustein (1987) have estimated that the Na^+/Ca^{2+} exchanger is capable of extruding Ca^{2+} from nerve terminals at a rate sufficient to match Ca^{2+} influx during stimulus trains at frequencies of up to 18/s. Thus, even the fastest component of increased release, the first component of facilitation, could conceivably be influenced by the action of the Na^+/Ca^{2+} exchanger.

The data presented in the preceding chapters support the hypothesis that the Na^+/Ca^{2+} exchanger specifically influences a shorter term component of increased release, the first component of facilitation. This finding lends support to the hypothesis that the different components of increased release represent the actions of different underlying biochemical or biophysical mechanisms (Magleby and Zengel, 1982). It is also consistent with the hypothesis of Katz and Miledi (1968) that one or more processes of increased release result from the accumulation of residual

calcium within the nerve terminal during repetitive stimulation.

The experiments presented in this dissertation support the hypothesis that a Na^+/Ca^{2+} exchange mechanism is involved in the first component of facilitation, but additional tests of this hypothesis would be desirable. For example, it would be desirable to have a specific inhibitor of the Na^+/Ca^{2+} exchanger in neuronal cells. The compound 3',4'-dichlorobenzamil (DCB), an analog of the Na^+/H^+ exchange inhibitor amiloride, has been reported to specifically inhibit Na^+/Ca^{2+} exchange in pituitary plasma membrane vesicles (Kaczorowski et al., 1985). However, DCB has been shown to have little or no effect on Na^+/Ca^{2+} exchange in the squid axon (Allen and Baker, 1986). Furthermore, amiloride and its analogs have recently been shown to inhibit Ca^{2+} influx through T calcium channels, an effect which could confound any effect mediated by inhibition of the Na^+/Ca^{2+} exchanger (Tang et al., 1988). A direct pharmacological test of the involvement of Na^+/Ca^{2+} exchange in processes of increased release awaits the development of more specific inhibitors of the exchange mechanism.

Contribution of Intracellular Calcium to Transmitter Release

Under conditions of low quantal content, evoked transmitter release appears to be largely determined by the third or fourth power of the concentration of extracellular

Ca^{2+} (e.g., Dodge and Rahamimoff, 1967). However, spontaneous quantal transmitter release can still occur under conditions of very low (less than 10 nM) concentrations of extracellular Ca^{2+}, which abolish evoked release (Miledi and Thies, 1971). Furthermore, stimulation-induced increases in miniature end-plate potential (MEPP) frequency can also occur under conditions of very low external Ca^{2+} concentrations (Miledi and Thies, 1971; Erulkar and Rahamimoff, 1978). These manifestations of quantal release are probably related to an effect of intracellular Ca^{2+}, since direct injection of Ca^{2+} into the presynaptic terminal of the squid giant synapse increases the spontaneous rate of transmitter release (Miledi, 1973). Additional evidence favoring a contribution of intracellular Ca^{2+} to spontaneous quantal release has been advanced by Erulkar et al. (1978), who showed at the frog neuromuscular junction that nerve stimulation under conditions of reversed electrochemical gradient for Ca^{2+} causes a reduction in MEPP frequency which is consistent with Ca^{2+} efflux from the nerve terminal during the action potential.

In this dissertation, evoked transmitter release has been shown to occur under conditions of very low concentrations of extracellular Ca^{2+} following pretreatment with ouabain, an agent known to result in an increase in intracellular Ca^{2+} concentrations (e.g., Deitmer and Schlue, 1983). The occurrence of this effect suggests that some mechanism exists to couple membrane depolarization to

transmitter release in the absence of sufficient amounts of extracellular Ca^{2+} to trigger release. Several hypotheses may be advanced, including a direct enabling effect of membrane potential on the release mechanism (e.g., Dudel et al., 1983), an effect of other intraterminal ions such as Na^{+} or Mg^{2+} on the release mechanism (e.g., Andreu and Barrett, 1980; Lev-Tov and Rahamimoff, 1980; Kharasch et al., 1981), and release of Ca^{2+} from intracellular stores (e.g., Blaustein, 1988). The latter effect would be consistent with the residual calcium hypothesis of Katz and Miledi (1968), which postulates that stimulation-induced increases in release result from a contribution of accumulated intracellular Ca^{2+} to the release process.

By analogy with skeletal and cardiac muscle, a process may exist in the nerve terminal which acts to couple membrane depolarization to Ca^{2+} release from intracellular stores. Calcium-induced Ca^{2+} release, presumably from sarcoplasmic reticulum, has been shown to occur in several systems including frog skeletal muscle (Endo, 1977), canine cardiac muscle (Fabiato, 1983), and barnacle muscle (Lea and Ashley, 1989). This release of Ca^{2+} can occur in the presence of very low triggering concentrations of extracellular Ca^{2+} (about 1 uM; Lea and Ashley, 1989). The smooth endoplasmic reticulum (SER) of the nerve terminal, an analog of the sarcoplasmic reticulum of muscle (Blaustein, 1988), is capable of sequestering considerable amounts of Ca^{2+} during periods of neuronal activity (Rasgado-Flores

and Blaustein, 1987a). Although depolarization does not normally trigger transmitter release in the absence of extracellular Ca^{2+} (del Castillo and Stark, 1952; del Castillo and Katz, 1954a), loading of the SER with Ca^{2+} (e.g., by pretreatment with agents which elevate cytosolic Ca^{2+} concentrations) could conceivably result in sufficient Ca^{2+} release from the SER to trigger evoked release in the presence of very low amounts of extracellular Ca^{2+}. The SER may be induced to release Ca^{2+} by a process of calcium-induced Ca^{2+} release in a manner analogous to that of Ca^{2+} release from muscle sarcoplasmic reticulum, or alternatively, by an inositol triphosphate-mediated mechanism (reviewed in Nahorski, 1988).

Significance

Understanding the mechanisms mediating the various properties of the process of neurotransmitter release will lead to increased appreciation of related mechanisms in many other biological systems. The mechanism of transmitter release in neurons may be analogous to the mechanism of exocytosis in other types of secretory cells such as exocrine pancreas, adrenal medulla, pituicytes, or mast cells, and the information on transmitter release presented in this dissertation may be applicable to the understanding of exocytosis in those systems. Furthermore, activity-induced modification of synaptic transmission has been proposed to play a major role in the processes of learning and memory (e.g., Hebb, 1949), and the results presented in

this dissertation may be of value in comprehending these important aspects of nervous system function.

Sustained elevations in intracellular Ca^{2+} concentrations are thought to contribute to the pathogenesis of hypoxic-ischemic neuronal injury such as that seen in acute stroke (reviewed in Choi, 1988). Although much attention has been focused on mechanisms of Ca^{2+} influx such as the cation channel activated by glutamate and N-methyl-D-aspartate (e.g., Rothman and Olney, 1986), other mechanisms of Ca^{2+} regulation such as the Na^{+}/Ca^{2+} exchange may also influence Ca^{2+} accumulation in injured cells. A greater awareness of the processes involved in regulating intracellular Ca^{2+} and transmitter release may lead to effective ways to limit the devastating neuronal damage caused by ischemic brain injury.

A greater appreciation of the processes underlying increased release at the neuromuscular junction will improve our understanding of the way in which neuromuscular transmission is affected by diseases of neuromuscular transmission such as myasthenia gravis and the Lambert-Eaton myasthenic syndrome, and by muscle relaxant drugs such as curare derivatives and acetylcholinesterase inhibitors which are used clinically.

Finally, the influence of the Na^{+} gradient on the magnitude of the first component of facilitation, a process which would be of greatest physiological significance during short trains of high-frequency stimuli, may suggest a

mechanism for the lowered seizure threshold seen in human patients when the serum Na^+ concentration is reduced below about 70% of the normal concentration (Adams and Victor, 1981). The reduction in the Na^+ gradient seen in clinically significant hyponatremia may be sufficient to reduce the homeostatic effect of the Na^+/Ca^{2+} exchanger on intracellular Ca^{2+} concentrations during neuronal activity. The resulting increase in intraterminal Ca^{2+} may increase transmitter release during bursts of action potentials in cortical neurons, thus predisposing networks of neurons toward the development of seizure activity.

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BIOGRAPHICAL SKETCH

Dennis R. Mosier was born in Corydon, Indiana, on September 11, 1962. His parents are Kenneth R. Mosier and Lillian V. Mosier. He received his grade school education in Brooksville, Florida, graduating from high school in May, 1980. In September, 1979, he entered the University of Florida, from which he received a B.S. degree in interdisciplinary medical sciences in May, 1983.

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After completion of his training at the University of Florida, he will undertake internship training in internal medicine at the Baylor College of Medicine in Houston, Texas. Subsequently, he will train in the specialty of neurology, also at the Baylor College of Medicine. He plans to enter a career in academic neurology, with emphasis on neurophysiological approaches to the study of synaptic transmission as it relates to nervous system disease.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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